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Mucoadhesive Films for the Buccal Delivery of Insulin

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Mucoadhesive Films for the Buccal Delivery of Insulin

by

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Dedication

To my family.

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Mucoadhesive Films for the Buccal Delivery of Insulin

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Co-Supervisor: Robert O. Williams

To address the need of a patient friendly and therapeutically effective method of administration of insulin (Ins) we sought to develop mucoadhesive films for delivery through the buccal mucosa. Ins is a labile molecule exhibiting limited activity and stability in solid solutions in films and other solid delivery devices. Early investigations outlined in Chapter 3 revealed the need for a certain particle size (below the one micrometer) for the addition of particulate material in films. In Chapter 4 a novel method for the manufacture of protein-coated nanoparticles (PCNP) is depicted. Successful particle batches were achieved in terms of size, uniformity, stability and activity and these particles were further investigated for their inclusion on films for buccal delivery. The method of manufacture of particles was based on an antisolvent co-precipitation process that immobilized macromolecules to the surface of crystalline core particles resulting in high yields and highly active protein loaded particles. Films loaded with PCNP were developed and characterized in Chapter 5. Lysozyme was utilized as a model macromolecule and high yields and activity were obtained after manufacture, demonstrating that after all the processing the protein is subjected to, activity is preserved. Using Eudragit® RLPO (ERL) as the matrix forming polymer, films with excellent mucoadhesion were developed. Here is described a high mucoadhesion for ERL

that was even further increased by the addition of the water soluble PCNP. This occurred by the water movement into the ERL matrix that the solubilizing particles generate. Finally, films containing Ins were developed and assayed for permeation through buccal mucosa. By adapting the method of manufacture, Ins-coated nanoparticles were obtained and embedded in films. ERL films corroborated previous findings by exhibiting excellent performance. Investigations on the permeation of Ins through buccal mucosa revealed that the inclusion of Ins in films enhanced its permeation in comparison with a control Ins solution. Thus here is described the successful development of mucoadhesive films for the buccal delivery of Ins.

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1. Introduction[1]

ABSTRACT

The buccal route of administration has a number of advantages including bypassing the gastrointestinal tract and the hepatic first pass effect. Mucoadhesive films are retentive dosage forms, and release drug directly into a biological substrate. Furthermore, films have improved patient compliance due to their small size and reduced thickness, compared for example to lozenges and tablets. The development of mucoadhesive buccal films has increased dramatically over the past decade because it is a promising delivery alternative to various therapeutic classes including peptides, vaccines, and nanoparticles. The "film casting process" involves casting of aqueous solutions and/or organic solvents to yield films suitable for this administration route. Over the last decade, hot-melt extrusion has been explored as an alternative manufacturing process, and has yielded promising results. Characterization of critical properties such as the mucoadhesive strength, drug content uniformity, and permeation rate represent the major research areas in the design of buccal films. This chapter will consider the literature that describes the manufacture and characterization of mucoadhesive buccal films.

[1] Significant parts of this chapter were taken from: J.O. Morales and J.T. McConville, Manufacture and characterization of mucoadhesive buccal films, European Journal of Pharmaceutics and Biopharmaceutics. (2011) 77, 187–199.

1.1. OVERVIEW

Films as dosage forms have gained relevance in the pharmaceutical arena as novel, patient friendly, convenient products. More recently, orally disintegrating films (or strips) have come to light thanks to their improved mechanical properties [1]. This translates into a less friable dosage form compared to most commercialized orally disintegrating tablets, which usually require special packaging [2]. Mucoadhesive buccal films share some of these advantages and more. Due to their small size and thickness they have improved patient compliance, compared to tablets [3–5]. Moreover, since mucoadhesion implies attachment to the buccal mucosa, films can be formulated to exhibit a systemic or local action [6]. Many mucoadhesive buccal films have been formulated to release drug locally in order to treat fungal infections in the oral cavity such as oral candidiasis [7–11]. Due to the versatility of the manufacturing processes, the release can be oriented either towards the buccal mucosa or towards the oral cavity, in this latter case it can provide controlled release via gastrointestinal (GI) tract administration. Alternatively, films can be formulated to release the drug towards the buccal mucosa. Films releasing drug towards the buccal mucosa exhibit the advantage of avoiding the first pass effect by directing absorption through the venous system that drains from the cheek [12]. Previously, many articles have reviewed the development of mucoadhesive buccal systems in global terms [13–17], or their specific attributes such as permeation enhancers [18] or mucoadhesive polymers [19–21]. The following is a review of the relevant literature which provides a background for understanding the rationale

behind the formulation of mucoadhesive buccal films, as well as reviewing the most crucial characterization techniques for these dosage forms. The reader should notice that the literature use the term film and patch interchangeably.

1.2. PHYSICOCHEMICAL PROPERTIES OF THE ORAL MUCOSA

The oral mucosa presents differently depending on the region of the oral cavity being considered [22]. The masticatory mucosa covers those areas that are involved in mechanical processes, such as mastication or speech, and includes the gingival and hard palate. This masticatory region is stratified and has a keratinized layer on its surface, similar to the structure found at the epidermis, and covers about 25% of the oral cavity [23]. The specialized mucosa covers about 15%, corresponding to the dorsum of the tongue, and is a stratified tissue with keratinized as well as non-keratinized domains [24]. Finally, the lining mucosa covers the remaining 60% of the oral cavity, consisting of the inner cheeks, floor of the mouth, and underside of the tongue. This lining epithelium is stratified and non-keratinized on its surface [25]. The buccal mucosa covers the inner cheeks and is classified as part of the lining mucosa, having approximately 40 to 50 cell layers resulting in an epithelium 500 to 600 μm thick (Figure 1.1) [26]. The epithelium is attached to underlying structures by a connective tissue or lamina propia, separated by a basal lamina. These lining mucosa and the lamina propia regions provide mostly mechanical support and no major barrier for penetration of actives [12,27]. The connective tissue also contains the blood vessels that drain into the lingual, facial, and

retromandibular veins, which then open into the internal jugular vein [12]. This is one of the main advantages of buccal over oral delivery: absorption through the buccal epithelium avoids the gastrointestinal tract conditions, such as gastric pH, enzyme content, and the first pass effect due to direct absorption into the portal vein. Once a given drug molecule reaches the connective tissue, it may be readily distributed, thus the permeation barrier is across the whole thickness of the stratified epithelium [12].

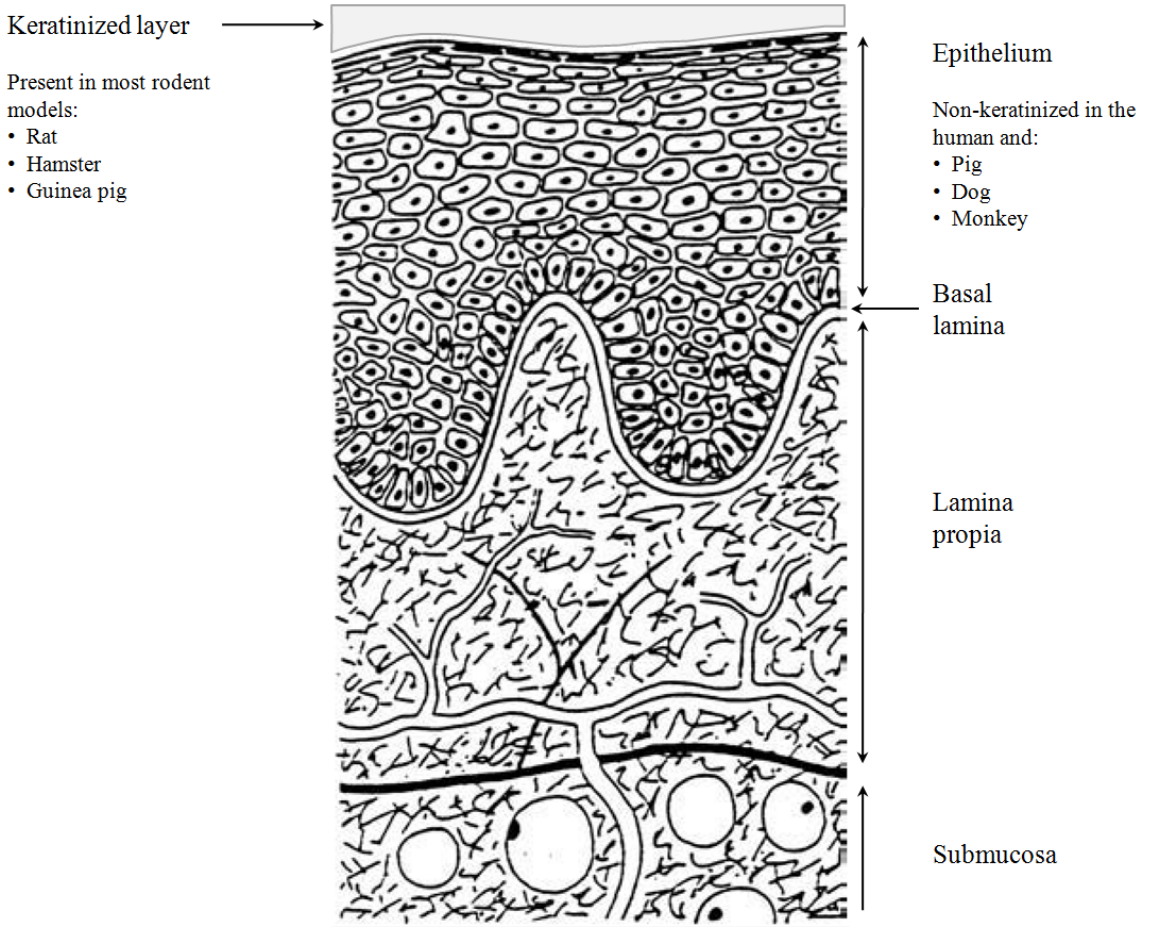

Figure 1.1. Diagram of a cross-section of the buccal mucosa. The keratinized layer is only present in most rodent models while the human has a non-keratinized buccal mucosa. Adapted from ref. [28].

The existence of membrane-coating granules in the epidermis has been well characterized and it is known to be the precursor of the keratin layer or stratum corneum [18,29]. Even though the existence of approximately 2 μm in diameter cytoplasmic membrane-coating granules in the buccal epithelium has been proven, less is known in terms of their function; however, the permeation barrier is believed to be related to the

presence of membrane-coating granules in the buccal mucosa [30,31]. Squier described these membrane-coating granules as organelles containing amorphous material that is extruded into the intercellular space after membrane fusion [30]. More recently, it has been reported that some of these granules also contain lipid lamellae domains organized to some extent [32]. This fact contrasts with the content of the membrane-coating granules in the epidermis, which contains very organized electron-dense lipid lamellae. Therefore, the intercellular space of the stratified non-keratinized buccal mucosa is filled with a combination of amorphous material presenting some domains where short stack of lipid lamellae can be observed. This important difference in the intercellular space composition is responsible for the difference in permeability between the buccal and keratinized mucosae for exogenous compounds [33].

Although the buccal mucosa is more permeable than keratinized epithelium, the existence of a permeability barrier has been described [34]. It was demonstrated that this barrier is located in the upper one third to one quarter of the epithelium layer using horseradish peroxidase, and by following its permeation through the epithelium. After topical application, the horseradish peroxidase only permeated through the first 1 to 3 cell layers. However, when injected subepithelially it was found to permeate through as deep as the connective tissue and up as far as the membrane-coating granules zone was [34]. This suggested that the permeability barrier is located in the upper region of the epithelium and is correlated with the rich lipid content of this zone. As well as the

keratinized epithelium, the intercellular space of the buccal mucosa is rich in lipids, but it is the difference in composition and the absence of the keratin layer that accounts for its permeation characteristics [33,35–38]. The lipid composition in the buccal epithelium has a higher content of phospholipids, cholesterol esters, and glycosylceramides, while the content of ceramides is minimal, compared to the skin and keratinized regions of the oral cavity [33]. This composition results in a higher concentration of polar lipids in the intercellular space [35]. Therefore, it is not only due to the highly organized lipid lamellae found in the keratinized epithelia, but also the nature of the lipid content that accounts for the increased permeation of the buccal mucosa compared to the skin and other keratinized epithelia.

Due to the polar nature of the lipids in the intercellular space, two different domains can be differentiated in the buccal epithelium: the lipophilic domain, corresponding to the cell membranes of the stratified epithelium; and the hydrophilic domain corresponding to the extruded content from the membrane-coating granules, into the intercellular space. These two domains have led to postulate the existence of different routes of transport through the buccal epithelium, namely the paracellular and the transcellular route [22]. The lipophilic nature of the cell membranes favors the pass of molecules with high log P values across the cells. Similar to the absorption mechanism in the small intestine, it is believed that lipophilic molecules are carried through the cytoplasm [18]. However, there still is a lack of evidence supporting this assumption. The

polar nature of the intercellular space favors the penetration of more hydrophilic molecules across a more tortuous and longer path [28,39,40]. It has been demonstrated that some hydrophilic molecules are subject to carrier-mediated transport through the buccal mucosa [41]. Most of the descriptions of molecules permeating through the buccal epithelium, in the literature, are related to the paracellular route of absorption. In an early study, it was found that tritiated water permeated through the paracellular route [37]. Using light microscopy autoradiography, it has been determined that water, ethanol, cholesterol, and thyrotropin release hormone, penetrate through the paracellular route as well [42,43]. More recently it was demonstrated using confocal laser scanning microscopy, that dextrans with 4 and 10 kDa average molecular weight and labeled with fluorescein-isothiocyanate, permeated through the paracellular route [44,45]. Even though there is no evidence that supports the idea of molecules permeating through the transcellular route, it is important to assess and understand the permeation route in order to determine strategies to enhance the absorption of actives when formulating buccal films.

1.3. FORMULATION AND MANUFACTURE OF BUCCAL DELIVERY FILMS

There are many factors in determining the optimum formulation of buccal delivery films, but three major areas have been extensively investigated in the mucoadhesive buccal film literature, namely mucoadhesive properties, permeation enhancement, and controlled release of drugs. Most of the polymers that are used as mucoadhesives are predominantly hydrophilic polymers that will swell and allow for chain interactions with the mucin molecules in the buccal mucosa [6]. Examples of these swellable polymers include hydroxypropyl cellulose (HPC), hydroxypropylmethyl cellulose (HPMC), hydroxyethyl cellulose (HEC), sodium carboxymethyl cellulose (SCMC), poly(vinyl pyrrolidone) (PVP), and chitosan; a full list of polymers used in the manufacture of buccal films, with additional descriptions and properties, is depicted in Table 1.1.

Table 1.1. Mucoadhesive and film forming polymers used in the literature

Mucoadhesive polymer in films	**Relevant properties and findings**	**Use in the literature**
Hydroxyethyl cellulose (HEC)	Non-ionic polymer High swelling properties and rapid erosion [46] Low mucoadhesive properties increased by the addition of SCMC [47] Zero order release kinetics of miconazole [46] and chlorpheniramine [48]	[46–51]
Hydroxypropyl cellulose (HPC)	Non-ionic polymer Increased swelling in ethylcellulose/HPC films [52] Moderate mucoadhesive properties [52,53] Zero order release kinetics of lidocaine [54] and clotrimazole [55] associated with erosion Square-root of time release kinetics of lidocaine [56]	[8,9,49,52–68]
Hydroxypropylmethyl cellulose (HPMC)	Non-ionic polymer Rapid swelling that plateaus [52] Moderate mucoadhesive properties [52,53,69] Initial burst followed by diffusion of nicotine hydrogen tartrate [70]	[4,46,47,50–53,56,69–84]
Sodium carboxymethyl cellulose (SCMC)	Anionic polymer High swelling properties that does not plateau [52] High mucoadhesive properties [47,52,78] Zero order release of miconazole nitrate [46] Diffusion governed release of ibuprofen [78]	[4,11,46,47,52,71,72,75,77,78,85–89]
Poly(vinyl pyrrolidone) (PVP)	Non-ionic polymer As film forming polymer exhibits non-fickian release of ketorolac [52] and progesterone [90] Used to tailor the release of propranolol [91] and miconazole [46] High swelling properties [90–92] Used as coadjuvant to increase mucoadhesion [78,93]	[46,49,50,52,75,77,78,80,86,90–97]
Poly(vinylalcohol) (PVA)	Non-ionic polymer Moderate swelling [73] and mucoadhesive properties [77,92] Anomalous release of miconazole [46]	[5,49,64,70,73,77,92]
Chitosan	Cationic polymer High to moderate swelling [47,98] and mucoadhesive properties [53,69,98,99] Sustained release of miconazole [46]	[10,46,51,53,69,74,81,82,90,92,94,95,98–108]

Table 1.1 continued

Alginate, sodium	Anionic polymer Rapid swelling and dissolution [47,104] High mucoadhesive properties [53]	[47,53,75,77,81,83, 104,109–111]
Agar	Poor and stable swelling properties	[104]
Carrageenan type λ	Poor and stable swelling and moderate mucoadhesive properties	[86]
Acacia	Very poor mucoadhesion	[69]
Guar gum	As an additive, conveyed moderate swelling and good mucoadhesive properties, and anomalous non-fickian release of miconazole	[51]
Poly-L(lactide-co-glycolide) (PLGA)	Micromatrices in buccal films to control the release of ipriflavone [101]	[101,112]
Polyacrilic acid, Carbopol®	Rapid, high and stable swelling [52,70,76,91] High mucoadhesive properties [53,69] As a film forming polymer, conveyed sustained release of buprenorphine [69] Used as an additive to tailor the release of propranolol [70,91]	[3–5,8,11,47,52,53, 57,69–72,76,77,79, 80,83,84,86–89, 91,93,105,110,113 –118]
Polycarbophil	Non-ionic polymer As an additive, conveyed moderate and stable swelling [86]and high mucoadhesive properties [47,56,58,86,119,120]	[9,47,56,58,70,86,1 19–122]
Poly(ethylene oxide)	Non-ionic polymer High mucoadhesion with high molecular weight [123,124] Zero order release kinetics of clotrimazole [123] and tetrahidrocannabinol [124] associated with erosion of the polymeric matrix	[56,123–125]
Poly(methacrylates)	Used as film former, exhibited very poor bioadhesive properties and low swelling capability [47,91,119] The salt form has high mucoadhesive properties [126]	[47,72,74,78,91,10 0,119–122,126, 127]

Table 1.1 shows that polymers from the families of the poly(acrylic acid) (Carbopols) and cellulosic derivatives have been extensively used as mucoadhesives, being part of the so called "first generation" mucoadhesives [128]. These polymers require to be hydrated in order to exhibit their mucoadhesive properties; however, a critical degree of hydration limits the phenomenon [129]. Above this critical value, over-hydration occurs leading to the formation of a slippery mucilage lacking mucoadhesive properties. In an early publication, Guo reported that the use of Carbopol® 934P alone exhibited the triple average peeling strength compared to the one exhibited by HPMC [69]. More recently, Semalty et al. demonstrated using a modified disintegration apparatus that the *in vitro* residence time of films formulated with a combination of Carbopol® 934P and HPMC E15 was almost the double than films containing only HPMC E15 [71]. Moreover, the combined polymers exhibited more resistance to rupture, as demonstrated using the the folding endurance test. Another important polymer widely used in the formulation of mucoadhesive films is HPC. In one of the earliest publication on mucoadhesive films, Anders and Merkle showed that the use of different grades of HPC or HEC had superior mucoadhesive properties compared to PVP and poly(vinyl alcohol) (PVA) as film forming polymers [49]. More recently, it was reported that film formulations, containing different ratios of Carbopol® and HPC exhibited longer *in vitro* residence times when the concentration of HPC was increased [57].

Natural and semi-natural polymers have also been reported in the literature as mucoadhesives. Chitosan was first introduced in 1994 by Guo for its use in mucoadhesive film formulations [69]. Following Carbopol® and HPMC as polymeric matrices for mucoadhesive films, chitosan exhibited better adhesion than acacia in a peeling test using an Instron 4201. In a more recent study, Shidhaye et al. described the manufacture, permeation, and mucoadhesive properties of chitosan films, containing gelatin and PVP in different proportions, for the buccal delivery of sumatriptan succinate [94]. It was demonstrated that an increase in the chitosan component increased the mucoadhesive strength of films. The authors attributed the increasing concentration of chitosan having the effect of increasing the number of amine groups that can interact with the negative charge groups (carboxyl, sulphate, etc.) which are present on the buccal epithelium surface [130]. Recently, mucoadhesive films have been developed and used as platforms for the oral delivery of nanoparticles [98,109]. Cui et al. reported on the manufacture of carboxylation chitosan-grafted nanoparticles (CCGNs) added to chitosan-ethylenediamine tetraacetic acid (C-EDTA) films with a backing layer of ethyl cellulose (EC) [98]. Films loaded with CCGNs exhibited higher mucoadhesion than that of placebo films. This high mucoadhesion effect was attributed to the high number of carboxyl groups that the CCGNs have, increasing the chance of hydrogen bonding with the mucosa [98].

It is evident that most of the mucoadhesive polymers explored in the literature are hydrophilic, or show some of the essential features for mucoadhesion. However, it has been reported that different insoluble Eudragit® grades can exhibit some mucoadhesive properties when used alone [72,100], or in combination with other hydrophilic polymers [47]. Films containing propranolol hydrochloride, Eudragit RS100, and triethyl citrate as a plasticizer exhibited almost three times the mucoadhesion force than that of films prepared with chitosan as the mucoadhesive polymer [100]. The authors proposed that the plasticizer is responsible for the increase on mucoadhesion. However, since the use of a plasticizer is necessary in Eudragit RS100 films, such film formulations may then be suitable for the manufacture of mucoadhesive dosage forms. Salts of soluble polymethacrylate derivatives, namely Eudragit S100 and L100, have been reported to increase mucoadhesion [126]. This study was based on the assumption that ionizable polymers exhibit the best mucoadhesive characteristics [131–133], which combined with low-swellable properties would allow for better patient compliance. It was demonstrated that, even though the Eudragit S100 and L100 did not exhibit mucoadhesive properties, their sodium and potassium salts performed equally or better than the positive mucoadhesive controls, namely Carbopol® 934P and HPMC [126].

The body of literature that explores different aspects of formulating mucoadhesive buccal films is extensive in terms of polymers used, mucoadhesive properties, and permeation characteristics for formulations. However, only a handful of products have

reached the market, and currently only two products for oral mucosal drug delivery have been successfully commercialized, and one further product has finished a phase 2 clinical study. BioDelivery Sciences International have used their BioErodible MucoAdhesive (BEMA™) technology platform to develop Onsolis™, a fentanyl buccal soluble film indicated to be administered in the buccal mucosa for the management of breakthrough pain in patients with cancer[134]. The formulation contains the mucoadhesive polymers carboxymethyl cellulose, hydroxyethyl cellulose, and polycarbophil, along with a backing layer to direct drug release towards the buccal mucosa. Using the same technology platform, BioDelivery Sciences International have completed a phase 2 clinical study for BEMA™ Buprenorphine with a significant improvement in the primary efficacy endpoint, SPID-8 (sum of pain intensity differences at 8 hours), compared to that exhibited by the placebo. The other commercialized film product is Suboxone™ Film, a buprenorphine and naloxone sublingual film. Using a polymeric matrix based on polyethylene oxide and hydroxypropylmethyl cellulose rapid dissolution and absorption are achieved [135].

The mucoadhesion process and the strategies used to control and enhance drug delivery and permeation will be discussed in later Sections 4 and 5. The following section will discuss the main manufacturing processes involved in making mucoadhesive buccal films, namely film casting and hot-melt extrusion.

1.3.1. Film casting

The film casting method is undoubtedly the most widely used manufacturing process for making films found in the literature. This is mainly due to the ease of the process and the low cost that the system set up incurs at the research laboratory scale. The process consists of at least six steps: preparation of the casting solution; deareation of the solution; transfer the appropriate volume of solution into a mold; drying the casting solution; cutting the final dosage form to contain the desired amount of drug; and packaging. During the manufacture of films particular importance is given to the rheological properties of the solution or suspension, air bubbles entrapped, content uniformity, and residual solvents in the final dosage form [136]. The rheology of the liquid to be casted will determine the drying rates and uniformity in terms of the active content as well as the physical appearance of the films. During the mixing steps of the manufacturing process air bubbles are inadvertently introduced to the liquid and removal of air is a critical step for homogeneity reasons [2]. Films cast from aerated solutions exhibit an uneven surface and heterogeneous thickness. Another recurrent concern in the manufacture of films for buccal delivery is the presence of organic solvents. The use of organic solvents is normally questioned, not only due to problems related to solvent collection and residual solvents, but also because organic solvents are undesired hazards for the environment and health [136]. However, due to the physicochemical properties of both the drug and excipients many formulations rely on the use of organic solvents, in which case they should be selected from ICH Class 3 solvent list [137]. Even though the

current literature on buccal films is mostly focused on platforms for specific drugs and diseases, manufacturing and processing parameters have been systematically reported. Examples of these research areas are related to the composition of the casting solution [53,96,102,118,140], drug concentration, the drug addition process, and cast solution rheology [86,87].

Since the early development of medicated films, content uniformity has been a major challenge for the pharmaceutical scientist. Schmidt proposed one of the earliest approaches to increase the drug uniformity of medicated films [138], by stating that the non-uniformity of films is inherent to their monolayerd nature. Schmidt proposed a multistep method for the manufacture of multilayerd films to overcome the heterogeneity of the monolayered form. However, Yang et al. reported that using the protocol proposed by Schmidt did not render uniform films [139], and went on to say that to overcome the non-uniformity of films, a manufacturing process for orally disintegrating films could be easily adapted for the manufacture of mucoadhesive buccal films. Yang et al. indicated that self-aggregation was one of the main reasons why films usually show poor uniformity, and in particular the drying process was found to be crucial in preventing aggregation or conglomeration of the ingredients of the film formulation [139]. During an inherently long drying processes, intermolecular attractive and convective forces are favored leading to the problem of self aggregation. In order to avoid non uniformity,

addition of viscous agents such as gel formers or polyhydric alcohols was proposed to alleviate potential self aggregation [139].

Recently, one of the main challenges in the film casting process, content uniformity along the casting surface, has been addressed [74]. Film characterization in terms of mucoadhesive, mechanical, permeation, and release properties have been widely investigated. However, prior to 2007 few reports pertaining to drug content uniformity can be found [70,86,99-101,141,151,153]. The most common approach to measure the content uniformity is the determination of drug by weight and not by casting area. Perumal et al. postulate that the determination by weight is erroneous because the final dosage form is determined by area instead of weight in the particular case of films. They demonstrate that custom made silicone molded trays, with individual casting wells for each dosage form, improved several characteristics significantly, including: the content uniformity per casting area unit, mucoadhesive properties, drug release, and thickness uniformity of monopolymeric or multipolymeric films [74]. Even though this approach may solve the problem of uniformity per dosage form, it does not guarantee the uniformity along the dosage unit itself, and also imposes limitations on scaling up possibilities.

1.3.2. Hot-melt extrusion of films

In hot melt extrusion, a blend of pharmaceutical ingredients is molten and then forced through an orifice (the die) to yield a more homogeneous material in different

shapes, such as granules, tablets, or films [140]. Hot-melt extrusion has been used for the manufacture of controlled release matrix tablets, pellets, and granules [141]; as well as orally disintegrating films [142]. However, only a handful of articles have reported the use of hot-melt extrusion for manufacturing mucoadhesive buccal films. Repka and coworkers have extensively conducted research on the use of hot-melt extrusion for the manufacture of mucoadhesive buccal films, evaluating different matrix formers and additives for the processing of the blend [9,56,58,59,123,124]. In an early publication, it was found that even though films containing exclusively HPC could not be obtained, the addition of plasticizers, such as PEG 8000, triethyl citrate, or acetyltributyl citrate allowed for the manufacture of thin, flexible, and stable HPC films over 6 months [60]. It has also been found that increasing the molecular weight of HPC decreases the release of hot-melt extruded films and allows for zero order drug release [55]. According to the models applied [143,144], the drug release was solely determined by erosion of the buccal film.

The most recent publications on mucoadhesive extruded buccal films involves the inclusion of Δ^9-tetrahydrocannabinol (THC) and its hemiglutarate ester prodrug (THC-HG) [58,124,125]. Successful mucoadhesive films could be obtained for THC at 120, 160, and 200°C while still containing at least 94% of the active ingredient. The greatest degradation into cannabinol was observed at 200°C (1.6%) [58]. For the formulation of the thermally labile prodrug THC-HG, the type of plasticizer was found to be crucial on

the post-processing stability [125]. The degradation of the drug in presence of PEG 8000, triacetin, or vitamin E succinate as plasticizers was found to be 1.7%, 1.1%, and 0.4% respectively, the latter being the most efficient plasticizer in preventing degradation at 90°C and 130°C [125].

1.4. MUCOADHESIVE AND MECHANICAL PROPERTIES OF BUCCAL FILMS

1.4.1. Overview of Mucoadhesion

Bioadhesion is the general term describing adhesion between any biological and synthetic surface. Mucoadhesion is a specific term describing the particular interaction of a mucosal membrane with a synthetic surface [145]. The phenomenon of mucoadhesion has been explained by applying any of the five theories of adhesion into the interaction of the dosage form and the biological substrate [13,145,146]. The reader is directed to detailed explanations of the electronic [147], adsorption [148,149], wetting [129,150], diffusion [129,151], and fracture theory [152], here we briefly summarize theories related to mucoadhesion theory. Since mucoadhesive buccal films include the interaction of a dry polymeric matrix that undergoes hydration, drug release, and sometimes erosion; the phenomenon is very complex. Smart has defined four possible scenarios for the analysis of the mucoadhesion process based on the hydration state of the dosage form and on the amount of mucus layer available for mucoadhesion [153]. Mucoadhesive buccal films can be classified as a "case 3" scenario since they are solid dry substrates that come in contact with a mucosa having thin or discontinuous mucus layers [153]. Relevant to the

analysis of the mucoadhesion of polymeric films on the buccal mucosa, are the adhesion theories of adsorption and diffusion. The adsorption theory states that the main contributors to the adhesive bond are the inter-polymer interactions, such as hydrogen bonds and van der Waals' forces [154]. The diffusion theory assumes that polymeric chains from the solid substrate, i.e. the mucoadhesive film, and the biological substrate, i.e. mucin in the mucosa layer, interdiffuse across the adhesive interface [145]. Important variables in this process are the diffusion coefficient of the polymer into the mucin layer and *vice versa*, the contact time, and the molecular chain length and their mobility [155,156].

Most of the mucoadhesive phenomena have two main stages that control the performance of the dosage form: the contact stage and the consolidation stage (Figure 1.2) [17,133]. Since mucoadhesive films are dosage forms that are brought in contact with the biological membrane by the patient, the contact stage is initiated by the patient. During the contact process the film will start dehydrating the mucus gel layer and will itself hydrate, initiating the interpenetration of the polymeric chains into the mucus and *vice versa*. For mucoadhesive films, which usually are designed to remain for prolonged times in contact with the buccal mucosa, a second stage, the consolidation stage, needs to take place in order to maintain this bond. In the consolidation stage the mucoadhesive strength will be determined by the polymer in the formulation, and how readily the dosage form hydrates upon contact with the mucus gel layer. This process is explained by

the dehydration theory, which explains that when a material capable of gelation, such as a mucoadhesive polymer in a buccal film, is brought into contact with an aqueous viscous colloid water will move until equilibrium is reached between the two layers [133,153]. The strength of the mucoadhesive bond will then be determined by the extent of intermixing that occurs after water migrates and reaches equilibrium.

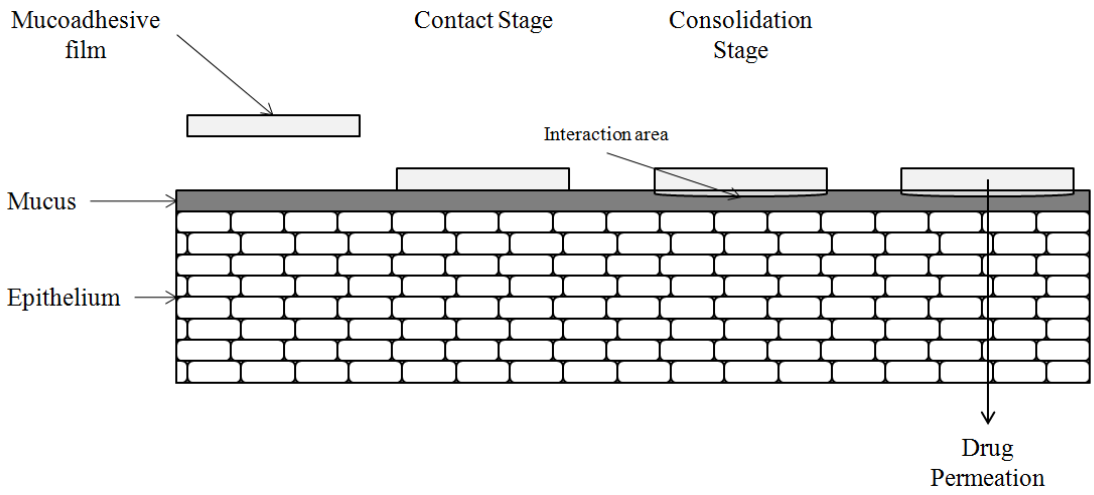

Figure 1.2. Contact and consolidation stages of mucoadhesion. Adapted from ref. [133].

Mucoadhesive films have been designed to remain in contact with the buccal mucosa for therapeutic purposes for prolonged periods of time. The measurement of the mucoadhesive strength and time of mucoadhesion have been described in parallel with formulation design since the very earliest publications in the field [49,69], this is further discussed in the following section.

1.4.2. Determination of mucoadhesion

The earliest approaches to measure bioadhesion were indirect and provided an idea of the trend that different formulations followed. In these experiments, instead of measuring the force of adhesion, the studies were focused on determining the time of adhesion or retention time of the dosage form in various models [8,49]. *In vitro* experiments usually consist of attaching a film to a glass plate, or to the sides of a beaker, and a mechanical force is applied either by moving the plate or by stirring the media in the beaker [46,76,119,122]. The first approach is normally done by modifying a standard USP disintegration apparatus [71]. In these experiments, a suitable substrate is attached to the surface of a glass slab, which is connected with the mobile arm of the disintegration apparatus. The film is then allowed to adhere to the substrate and the time necessary for complete erosion or detachment is recorded as the *in vitro* residence time [46]. Conditions such as the medium composition, pH, temperature, salts addition, or nature of the substrate can be controlled [77] and will modify the results, hence it is important to report the conditions used to obtain reproducible data [11,90,92,94]. The second approach often used in the literature requires the adhesion of the film into a static surface, normally the side of a beaker, and detachment force is applied by the stirring media [76]. Modifications of this approach include the adhesion of a biological substrate to the side of the beaker, normally a non-keratinized tissue layer such as porcine buccal mucosa [78] to further mimic the physiology of the human buccal epithelium. Again, controlling the

composition of the media, temperature, pH, or the nature of the substrate (either from a biological or synthetic source), will determine the final mucoadhesion or *in vitro* residence time [47,90,91,110].

Even though the measurement of the *in vitro* mucoadhesion or residence time provides information to optimize formulations, it does not elicit the real strength of the mucoadhesive bond. The first article to report on a peeling test for mucoadhesive buccal films was published by Guo in 1994. In these experiments a load cell is attached to the mobile section of the instrument and force of detachment is obtained and plotted against either distance or time. The mucoadhesion strength of films formulated with Carbopol® 934P, HPMC, chitosan, or acacia gum was expressed as the maximum peeling strength or load using an texture analyzer, such as the Instron 4201 [69]. After this publication a number of other articles reported on the use of tensile testing instruments, such as the Instron for the measurement of bioadhesive properties. Li et al. were the first to publish the use of a biological membrane as the adhesive substrate for measuring mucoadhesion of buccal films [3]. Freshly excised rabbit buccal mucosa was glued onto a stainless-steel platform. Likewise, a buccal film sample was attached to another platform, and following the addition of a drop of water, the film and the substrate were allowed to adhere for a predetermined amount of time. The mucoadhesion strength was measured as the maximum applied force needed in order to detach the film from the substrate [3]. The development of the bench top texture analyzer that allowed for accurate measurement of

very small variations, as well as being able to control the contact force and time, increased the number of publications that reported on mucoadhesion and tensile properties of buccal films. The first report on the use of the TA.TX2® texture analyzer (Stable Micro Systems) to measure the mucoadhesion strength of buccal films utilized chicken pouch as the biological membrane upon which the films were allowed to adhere [72]. The instrument measures detachment forces from its mobile arm, which after normalizing is considered as adhesive forces and the maximum force is normally referred to as mucoadhesive force. The use of this type of texture analyzer for the measurement of mucoadhesion on different dosage forms, such as buccal tablets had already been published [102,157]. This previous research had focused on the importance of the method variables, which ultimately determine, together with the film and the substrate properties, the value of mucoadhesion strength [157]. Using the instrument depicted in Figure 1.3, the authors demonstrate that contact force, contact time, and the speed of probe withdrawal during the mucoadhesion experiment, all affect the experimental outcome. The contact time and contact force represent the effort the patient needs to provide in order to bring the dosage form in contact with the buccal mucosal surface, and allow for mucoadhesion at the "contact stage" mentioned previously. The contact time was found to be more critical in the affecting the mucoadhesive strength than the contact force. With the exception of an increase in contact time from 10 to 30 seconds, increasing the contact time significantly increased the measured mucoadhesive strength. However, the authors

demonstrated that increasing the contact force from 0.05 N to 0.1 N, or 0.5 N to 1.0 N did not significantly increase the mucoadhesive force [157]. Since the development of the mucoadhesive strength test by Wong et al., several modifications have been used to determine this parameter on buccal films [7,56,58,70,78,86,100,123,124]. Measuring the peak force needed to detach a biological substrate attached to a mobile probe, from a wetted mucoadhesive film at various sections of a film cast surface, has been used to demonstrate that the texture analyzer can be used to determination of mucoadhesion uniformity along the casting area [9]. This is particularly important since the few articles published in the literature account only for the drug content uniformity, and do not report any uniformity assessment of film functionality [74]. Some other approaches to measure mucoadhesion include the modification of different mass balance apparatuses to determine the detachment force from the mucoadhesive joint between the buccal film and usually a biological substrate [75,79,88,91,93–95,98,109,110]. The reader should note that there is no standardized mucoadhesion test in the literature, which makes the experimental conditions different from paper to paper so extrapolation and comparison of results should be cautious. Moreover, methods that rely on excised tissue are prone to exhibit larger standard deviations compared to *in vitro* conditions.

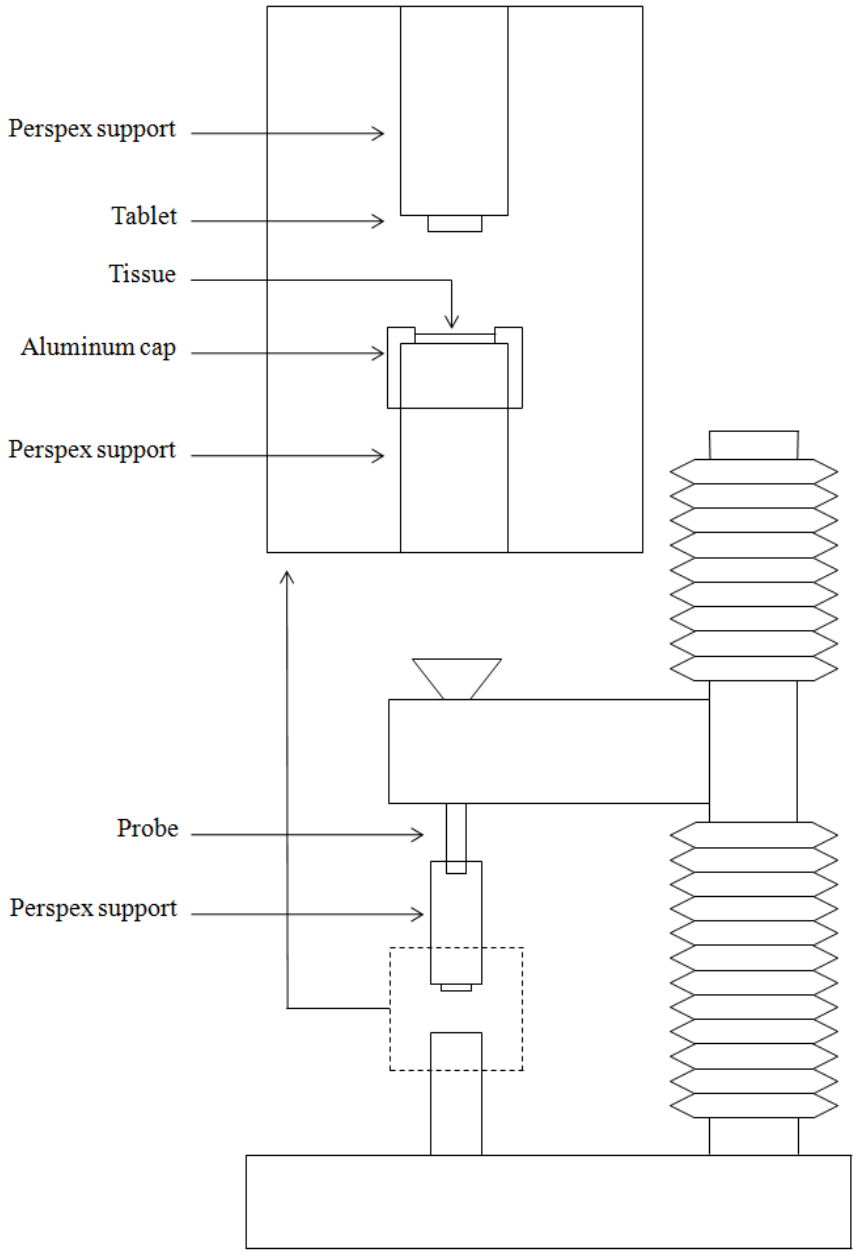

Figure 1.3. Mucoadhesion testing apparatus using the texture analyzer TA.XT2, modified from ref. [157].

1.4.3. Determination of mechanical properties of mucoadhesive films

Besides the important parameter of mucoadhesion strength and residence time of buccal films, the mechanical properties play a crucial role on the physical integrity of the dosage form [4]. Several values can be obtained from a regular stress-strain curve; however, most relevant to the study of buccal films are the tensile strength, the elongation at break, and the elastic modulus, also known as Young's modulus [4,158]. The determination of the mechanical properties of a buccal film is usually based on the ASTM D882 method [159] and measured using instruments such as a texture analyzer. The tensile strength of a film is defined as the resistance of the material to a force tending to tear it apart [60–62,94,99,100,103,123,160], and normally identified as the maximum stress in the stress-strain curve and it can be computed in accordance to Equation (1)[161].

$$Tensile\ strength = \frac{Force\ at\ failure}{Cross-sectional\ area\ of\ the\ film} \quad (1)$$

The elongation at break is a measurement of the maximum deformation the film can undergo before tearing apart and is calculated using Equation (2):

$$Elongation\ at\ break = \frac{Increase\ in\ length\ at\ break}{Initial\ film\ length} \times 100 \quad (2)$$

In general, elongation (or strain) will increase with an increasing content of suitable plasticizing agents in a given formulation [162].

Young's modulus is an evaluation of the stiffness or how the film deforms in the elastic region [142]. It is defined in the initial elastic phase of deformation and is obtained from the ratio of applied stress and corresponding strain and can be computed from the slope of the stress-strain curve using Equation (3):

$$Young's\ modulus = \frac{Slope\ of\ stress - strain\ curve}{Film\ thickness \times Cross - head\ speed} \quad (3)$$

It has been described that soft and weak polymers have a low tensile strength, Young's modulus and elongation at break; while a soft and strong polymer exhibits a moderate tensile strength, low Young's modulus, and a high elongation at break [163,164]. Desired mechanical properties will vary depending on the formulation goals and the method chosen, but in general some examples of behaviors obtained from stress-strain curves can be depicted, as shown in Figure 1.4 [162].

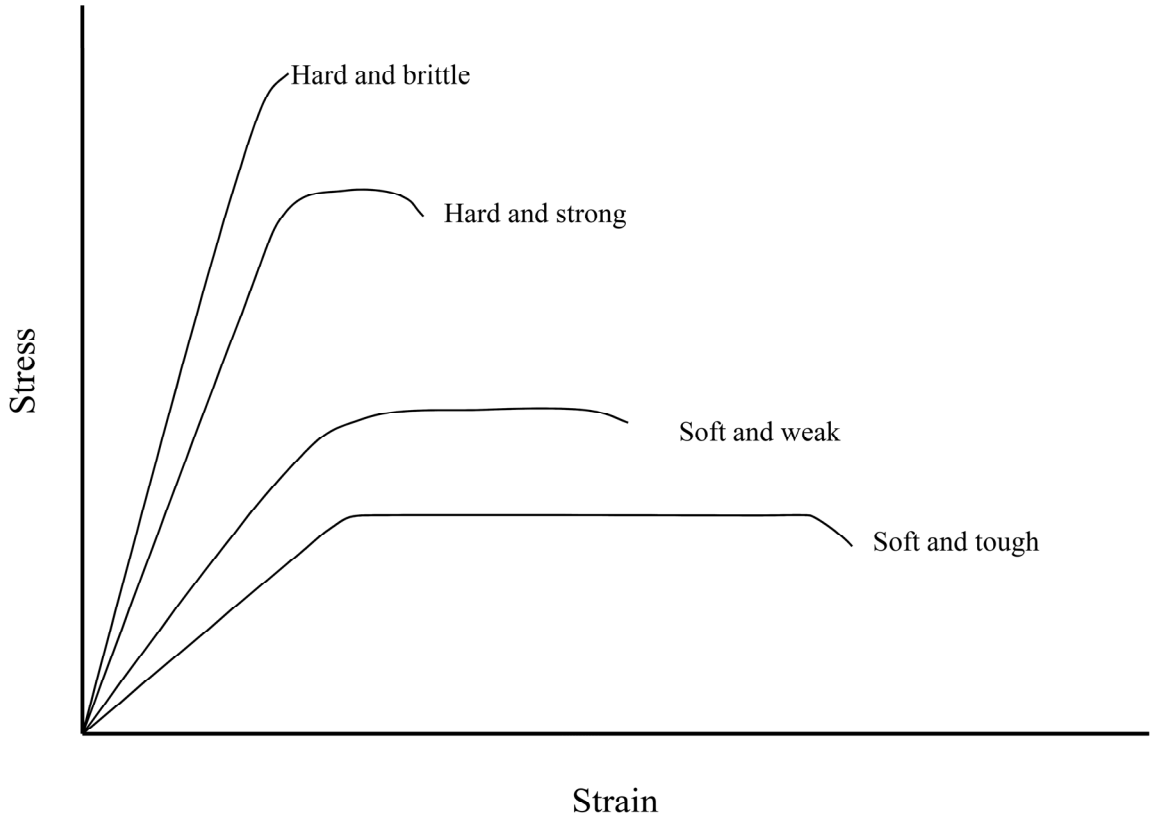

Figure 1.4. Examples of behaviors observed in stress-strain curves in polymeric films (Adapted from ref. [162]).

Tear resistance of a film is normally obtained from stress-strain curves but using very low rates of loading (displacement of 51 mm/min). It is a complex function of the film ultimate resistance to rupture and is obtained from the maximum stress value and is reported as the correspondent force [2,165].

Finally, another test normally used and reported in the literature, is the determination of the folding endurance of the film. The test is performed by repeated folding the film at the same place until film failure [95]. A maximum of 300 times is

sometimes reported as a limit to the test [166], and the value is reported as the number times the film can be folded prior to rupture.

1.5. ASSESSMENT AND ENHANCEMENT OF PERMEATION THROUGH THE BUCCAL MUCOSA

1.5.1. Permeation rate determination

Since the early research on the development of mucoadhesive buccal films, drug release from polymeric matrices have been well characterized and reported [54,69,113]. However, these studies were usually conducted using standard or modified dissolution apparatus, thus obtaining only an estimate of the rate of drug release from the film and not penetration rates through the buccal mucosa. Although it is well known that the bioavailability of drugs administered through the buccal route can be highly impacted by the permeation rate through the biological membrane, *in vitro* characterization of permeation properties has not been addressed until recently [7,52,71,77,80,85,91,94,96,110,114]. The experimental procedure typically involves the use of a diffusion cell, which can be either vertical, such as a Franz diffusion cell, or horizontally oriented, such as the side-by-side or Ussing diffusion cell. In these cells a donor compartment is separated from a receptor compartment by a membrane acting as the mucosa model. Conditions such as temperature, composition of the receptor and donor media, pH, cell dimensions, and hydrodynamic conditions are normally controlled in these experiments.

One of the most important components is the membrane that separates the donor from the receptor chambers. It is reported that these membranes may come from either synthetic or biological sources. Synthetic membranes provide a consistent porous path and thus can be used to effectively performance rank different formulations [50]. As a substitute for excised animal buccal mucosa, synthetic membranes decrease the large sample to sample variation due to the high structural homogeneity they exhibit, compared to *ex vivo* methods [167,168]. However, their use is limited due to the absence of a stratified non-keratinized epithelium that is present in the buccal mucosa. Depending on the physicochemical characteristics of the drug, permeation of different absorption pathways and interactions with the epithelium will be found, all of which are not applicable in synthetic membranes [168]. Thus, freshly excised mucosa is widely used as the barrier membrane in diffusion studies since it most closely resembles the *in vivo* permeation scenario [27,169]. Consequently, the selection of the animal model is of high importance due to the anatomical differences observed in buccal mucosa among species. Even though rodents are normally the first choice as animal models, their use for buccal delivery purposes is very limited due to their keratinized buccal membrane (Figure 1.1). The best model among rodents is the rabbit due to its para-keratinized buccal membrane [170]. In general, large animals exhibit a non-keratinized stratified buccal mucosa, which is more similar to the anatomy of the human, and is differentiated mostly in the thickness and permeation properties of the tissue [12]. In terms of availability, thickness, and

permeation properties, the swine buccal mucosa appears to be the most suitable animal model, and this is demonstrated widely in the literature [71,77,94,96,110]. Other potential models to study buccal permeation are the dog and the monkey but due to availability, cost, or ethical concerns they are not commonly used in buccal film research [12]. Another crucial consideration for the permeation test is the tissue storage and isolation before the experiment. This is often overlooked and not reported hindering an adequate interpretation of results. Even though the permeation barrier is believed to be located in the upper one third or one quarter of the epithelium, the connective tissue should be removed before the test in order to prevent differences in permeating path, which could translate into large sample to sample variations [169]. The use of chemical, thermal, and enzymatic treatments for removing the connective tissue are not considered to be the methods of choice, as they have either demonstrated to alter permeation, or are topics of debate as to their applicability [169,171]. Thus, surgical removal of the connective tissue is the preferred treatment and it is normally performed right before the permeation experiment by soaking the tissue in phosphate buffer at controlled pH [52,94,110,114]. Integrity of the epithelium before and after the experimental procedure is desired, and it is normally determined by measuring permeation of a non-permeating molecule [172,173] or by light microscopy [114,169].

From a more practical standpoint, the permeability coefficient (*P*) and the diffusion coefficient (*D*) are derived from the diffusion process described in one dimension by Fick's second law transformed and simplified to Equation (4) [169].

$$F = \frac{P \cdot S}{V_D}\left[t - \frac{h^2}{6D}\right] \quad (4)$$

Where the fraction of drug transported (*F*) is obtained from the relationship between *P*, the surface area of the tissue (*S*) (the opening of the diffusion cell), the volume of the donor compound (V_D), time (*t*), the effective length of buccal mucosa the drug must traverse (*h*), and *D*. During a standard diffusion study, *P* is obtained from the slope of the fraction permeated versus time curve while *D* is calculated from the x-axis intercept as seen in Equation (4).

1.5.2. Permeation enhancers in mucoadhesive buccal films

Due to the limited permeability of the buccal mucosa compared to that of the intestinal epithelium, the use of permeation enhancers has been widely investigated in dosage forms for buccal delivery [18]. Permeation enhancers are pharmaceutical ingredients included in a formulation in order to improve the permeation characteristics of the drug through the target mucosa and are desired to demonstrate null or very limited toxicity or tissue damage [174]. It is known that permeation through the buccal epithelium occurs either by the transcellular or paracellular route as previously described,

but in general the overall process can be considered to be governed by passive diffusion [175] and modeled by Fick's first law of diffusion, as shown [18] in Equation (5):

$$J_{ss} = \frac{D \cdot K}{h} \cdot C_D \quad (5)$$

Where, the steady state flux (J_{ss}) is determined by D of the drug within the buccal mucosa, the partition coefficient (K) of the drug between the buccal mucosa and the donor chamber solution, the concentration of drug in the donor compartment (C_D), and h. Most of the permeation enhancers will alter the mucosa or the permeating molecule in such a way that D or K or both can be enhanced. Based on the physicochemical characteristics of the drugs, delivery using different permeation enhancers will be suitable. As seen in Table 1.2, permeation enhancers used in the buccal mucosa favor the paracellular route of drug absorption, or it has been suggested that they work in such a way. Even though many permeation enhancers have been described to be effective in the buccal mucosa [176–179], few buccal film formulations have studied the inclusion of such agents in their compositions. One of the earliest studies with penetration enhancers in the formulation included the use of glycyrrhizic acid in lidocaine containing HPC mucoadhesive buccal films. In this study, the authors found a direct relationship between an increase of glycyrrhizic acid content and the penetration rate through freshly excised hamster buccal epithelium. Thus in this example, a permeation enhancement effect of glycyrrhizic acid in the presence of the active ingredient was seen through the keratinized rodent epithelium [63]. In a previous report that same authors hypothesized that the effect

of glycyrrhizic acid is due to the formation of an amorphous state of lidocaine in the dosage form [54]. In another study, oleic acid and propylene glycol monolaurate were used as penetration enhancers for lidocaine hydrochloride in mucoadhesive buccal films made of Carbopol® 971P. The permeation studies for these lidocaine films were performed using Franz diffusion cells, and utilizing porcine buccal mucosa as the model membrane. After demonstrating that oleic acid as the penetration additive exhibited the best enhancing characteristics, the authors performed *in vivo* studies and proved that incorporation of oleic acid did not produce any discernible redness or irritation of the buccal mucosa after 8 hours of exposure [114]. More recently, films formulated with chitosan and PVP K30 as mucoadhesives with different permeation enhancers were tested to determine the highest increase in diffusion of sumatriptan succinate through porcine buccal mucosa [94]. It was determined that the use of dimethyl sulfoxide in the highest concentration studied (3% w/w) exhibited the best enhancing characteristics compared to transcutol 5% w/w or polysorbate-80 1% w/w. The addition of penetration enhancers did not modify the physicochemical properties of their formulations, making them ideal for the manufacture of improved mucoadhesive buccal films.

Table 1.2. List of permeation enhancers used for buccal delivery.

Permeation enhancer	Proposed mechanism of action	Preferred route enhanced	Examples
Surfactans	Lipid extraction from the mucosa	Paracellular	Sodium dodecyl sulfate [180] Sodium lauryl sulfate [181]
Bile salts	Lipid extraction from the mucosa	Paracellular	sodium glycocholate [45,182] sodium taurocholate, sodium glycodeoxycholate, and sodium taurodeoxycholate [45] sodium deoxycholate [85]
Fatty acids	Increase fluidity of intercellular lipids	Paracellular*	Oleic acid [114,183,184] eicosapentaenoic acid and or docosahexaenoic acid [184]
Ethanol	Disrupt arrangement of intercellular lipids	Paracellular*	[185,186]
Chitosan	Increase retention time of drug in contact with mucosa and disruption of intercellular lipid organization	Paracellular*	

* No definitive evidence

1.6. CONCLUSION

The buccal mucosa is a promising delivery route for drugs that need to avoid the gastrointestinal tract due to degradation by the gastric pH, intestinal enzymes, or due to a substantial hepatic first pass effect. It can also be an alternative to skin, pulmonary, or nasal delivery. The physiology of the buccal mucosa allows for the penetration of active substances and due to its rapid cellular turnover and recovery, the use of penetration enhancers is possible. Moreover, recent publications have proved that the addition of permeation enhancers on buccal films did not hinder the manufacturing capability nor imposed mucosal irritation or toxicity. In the lab scale, film casting remains as the manufacturing process of choice. Nonetheless, hot-melt extrusion has been successfully explored as a method for obtaining mucoadhesive buccal films for the delivery of THC through the buccal mucosa. Many possibilities remain in the design of buccal films, including their recent application as platforms for the delivery of nanoparticles. In this dissertation the use of particulate material in films is described. Insulin-loaded nanoparticles were developed and the physicochemical implications they impose on films were investigated and are depicted below.

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2. Research Outline

2.1. Overall Objective

In this dissertation it was hypothesized that films could be manufactured to contain insulin in a stable, active, and uniformly distributed form for its buccal delivery. Insulin was sought to be formulated into particles in such a way that it would keep its activity and high yield at the end of the process. For this, the goal of this project was to establish both a polymer platform (i.e. a film) that could serve as a buccal solid vehicle for macromolecular actives (e.g. insulin). This research sought to study a myriad of materials for the manufacture of films for buccal delivery. This work describes, in contrast to what has been assumed in the literature, some hydrophobic materials such as polymethacrylates exhibit excellent mucoadhesion, and they were further explored in conjunction with the particle manufacturing process. In that regard, another objective was to develop a nanoparticle manufacturing process for a variety of macromolecules. For this goal, bovine serum albumin (BSA), lysozyme (Lys), and insulin (Ins) were studied as models to render high yields, high activity, and narrow nanoparticle polydispersity.

2.2. SPECIFIC OBJECTIVES

2.2.1. Development of Water-Swellable Polymethacrylate Mucoadhesive Buccal Films Containing Caffeine Particles

After preliminary work on several materials for the manufacture of films for buccal delivery, it was sought to further investigate the polymethacrylate derivatives commercialized as Eudragit® RLPO (ERL) and RSPO (ERS). We successfully developed films with adequate mechanical properties evaluated *in vitro* by analyzing stress versus strain profiles of film strips were successfully developed. Also for the first time ERL was described as being an excellent mucoadhesive polymer *in vitro*, especially by comparing its performance with typically referred to as mucoadhesive polymers in the literature, such as Carbopol® 974P and polycarbophil. By imaging cross sections by scanning electron microscopy of the films, the ultrastructure was explored and a correlation between size of caffeine particles and mucoadhesive and mechanical performance was established. Furthermore, various release profiles could be achieved with all formulations studied, more specifically for ERS. Caffeine release was found to be governed by first order release kinetics and also affected by the size of caffeine particles. Thus we were able to manufacture films for buccal delivery and also establish a particle size cutoff for optimal mechanical, mucoadhesive, and release performance.

2.2.2. A Design of Experiments to Optimize a New Manufacturing Process for High Activity Protein-Containing Submicron Particles

To incorporate macromolecules in the films that we had obtained, we sought to develop a novel manufacturing process for high activity and yield of macromolecule-containing submicron particles. The need for such particle size had been described in the previous chapter based on the physicochemical properties that particles impart to films. Based on an antisolvent co-precipitation process, the effect of various mixing techniques and various means of adding the aqueous phase into the organic phase were studied. The effect of a variety of organic solvents as the antisolvent phase; type and amount of the amino acidic core material of particles; and the use of surfactants both in the aqueous and the organic phase were also investigated. Ultimately, it was found that the combined use of high energy mixing by means of a sonicator, stabilizing surfactants in the organic phase, and increased surface area for addition of the aqueous phase by means of nebulization allowed for submicron sized and nanosized batches of particles coated with either BSA or Lys on crystalline cores of D,L-valine. To further optimize the novel method, multiple designs of experiments (DoE) were performed in order to define the critical processing parameters. Optimized conditions allowed for high yields, high activity, as well as control over polydispersity of submicron sized particles.

2.2.3. Protein-coated Nanoparticles Embedded in Films for Buccal Delivery

As initially planned, it was sought to combine both research efforts into protein-loaded films for buccal delivery. The need for the addition of the macromolecules in a

more stable form compared to solid solution was sought to be addressed with the use of the novel method of manufacture of nanoparticles. This method was further optimized at this stage by studying the controlling effects of pH over size and polydispersity of protein-coated nanoparticles (PCNP). High yield and high activity Lys-containing particles were obtained and further formulated into films for buccal delivery. By using ERL as the film forming matrix, PCNP-loaded films were successfully manufactured and evaluated for their physicochemical properties. Excellent mechanical and mucoadhesive properties were achieved in ERL films and Lys release was found to be tunable by the addition of the water-swellable and soluble HPMC. Sustained release was observed over four hours and full activity of Lys was retained during this period of time.

2.2.4. Development of Films of Insulin-Coated Nanoparticles for use in Buccal Delivery

Finally to achieve the goal of this dissertation, films for buccal delivery of insulin were developed. The method of particle manufacture was adapted to obtain Ins-coated nanoparticles (ICNP) with high yield, stability upon storage, and narrow polydispersity. These particles were then formulated into films for buccal delivery applications. Films were characterized for their mechanical and mucoadhesive performance and ERL was found to render films with optimal properties. Ins release was found to be similar for ERL and ERL-HPMC combinations and the films were further analyzed for permeation through a human buccal mucosa model. Three dimensional models of human model mucosa obtained by differentiation of normal human keratinocytes were used to study the

permeation characteristics of the Ins-loaded films. Franz diffusion and well plate diffusion studies were conducted and an enhancement effect could be evidenced by including Ins in films as solid dosage forms in comparison to a control solution of insulin.

3. Development of Water-Swellable Polymethacrylate Mucoadhesive Buccal Films Containing Caffeine Particles[2]

ABSTRACT

The aim of this work was to investigate the influence of particles on the properties of polymethacrylate films intended for buccal delivery. A solvent casting method was used with Eudragit RS and RL (ERS and ERL, respectively) as film forming rate controlling polymers, with caffeine as a water soluble model drug. The physicochemical properties of the model films for a series of formulations with increasing concentrations of caffeine were determined in terms of morphology, mechanical and mucoadhesive properties, drug content uniformity, and drug release and associated kinetics. Typically regarded as non-mucoadhesive polymers, ERS and mainly ERL, were found to be good mucoadhesives, with ERL01 exhibiting a work of mucoadhesion (WoA) of 118.9 μJ which was about 5-6 times higher than that observed for commonly used mucoadhesives such as Carbopol 974P (C974P, 23.9 μJ) and polycarbophil (PCP, 17.4 μJ). The mucoadhesive force (MAF) for ERL01 was found to be significantly lower yet comparable to C974P and PCP films (211.1 vs. 329.7 and 301.1 mN, respectively). Inspection of cross-sections of the films indicated that increasing the concentration of caffeine was correlated with the appearance of recrystallized agglomerates. In conclusion, caffeine agglomerates had detrimental effects in terms of mucoadhesion, mechanical

[2] Significant portions of this chapter were taken from: J.O. Morales, R. Su, J.T. McConville, The Influence of Recrystallized Caffeine on Water-Swellable Polymethacrylate Mucoadhesive Buccal Films, AAPS PharmSciTech. (2012) *In Press*.

properties, uniformity, and drug release at large particle sizes. ERL series of films exhibited very rapid release of caffeine while ERS series showed controlled release. Analysis of release profiles revealed that kinetics changed from a diffusion controlled to a first order release mechanism.

3.1. INTRODUCTION

The development of films as mucoadhesive dosage forms for buccal delivery of actives is a field that continues to grow due to unique characteristics that are advantageous for drug delivery [1–3]. In physical terms, films may be preferred over tablets due to size, flexibility and comfort [1]. As adhesive dosage forms, films can be formulated for a variety of delivery regimens as well providing the opportunity for locally treating diseases by direct application. The buccal route also offers interesting advantages over the oral route mainly for molecules that could be rendered inactive through the gastrointestinal tract, i.e. peptides and proteins. In addition, rapid absorption and peak concentration can be elicited through the venous system that drains from the cheek [4].

Most mucoadhesive films for buccal delivery are manufactured by the solvent casting technique regardless of the growing body of literature describing film manufacture by hot-melt extrusion [5–8]. The solvent casting technique is scalable, simple to execute, and cost-effective in the laboratory scale [3]. However this method of manufacture is limited by environmental concerns, due to the use of organic solvents, and additionally long processing times that can impose budget limitations [8]. The solvent casting technique involves the solubilization or dispersion of all the ingredients in a suitable solvent system and then controlled drying to yield the drug-containing films. Arising from manufacturing challenges, a recent publication has surveyed the literature

regarding drug content uniformity and revealed the lack of reports addressing this issue [9], which is a basic yet an utterly important variable in film manufacture. In the manufacture of films, cast sheets are cut into unit doses which could result in high variability of drug content if this is not addressed adequately during the developmental stages of the formulation. The main concern raised in the literature is the appearance of agglomerates upon drying of films [10]. This was attributed to long drying times that allow for attractive forces between molecules to build up and result in the formation of agglomerates and was dealt with the addition of viscosing agents that could prevent agglomeration during drying. In an alternative to this strategy, Perumal et al. (2008) created casting trays that would allow for the manufacture of unit doses without the need to cut strips from a cast sheet. Even though, this method improves results in terms of content uniformity it does not address uniformity among the surface of the single unit, and it could be impractical for scaling up purposes [9].

Several excipients can be used to control for different properties of the films. Usual materials can include but are not limited to film forming polymers, mucoadhesive polymers, backing polymeric layer, plasticizers, taste masking or sweetening ingredients, stabilizers, and rate controlling polymers [3,11,12]. However, the polymer system that controls the release of the active is one of the most prominent areas of development of films. Most recent reports on the use of polymethacrylates as film forming polymers feature them mainly as a drug controlling materials in the formulation [13–15]. In these

studies, Eudragits have either been part of the drug containing layer or as part of the release rate controlling layer. Only few articles have described the use of Eudragits as a mucoadhesive material [16,17]. Eudragit RS (ERS) and Eudragit RL (ERL) are polymethacrylates possessing a quaternary ammonium group branching out of their polymer backbone. The presence of these cationic groups allow for water permeability, resulting in swelling of the polymer matrices. In a systematic comparative study, both ERS and ERL were found to be non-mucoadhesive materials with very low adhesion, similar to that determined in the same study for alginic acid and chitosan [18], both of which are normally considered mucoadhesive materials [19]. Conversely, a more recent publication by Perumal et al. (2008) has shown that ERS films can elicit high mucoadhesive properties measured both in terms of maximum detachment force and work of adhesion [17]. Moreover, films containing only ERS exhibited increased mucoadhesive properties compared to those found in ERS-chitosan films. On another study, ERL was found to be the least mucoadhesive material and the polymer that showed the lowest swelling capacity in comparison to HPMC-E15, sodium carboxymethyl cellulose (SCMC), and Carbopol 934P (C934P). However, in the same study the in vitro residence time was found to be 1.75 hours, comparable to that observed for HPMC-E15 [20]. One investigation that utilized Eudragit L100 (EL100) and S100 (ES100) as mucoadhesive materials required prior modification into sodium and potassium salts [16]. The modified salt form was used to enhance the mucoadhesive

properties of these polymethacrylates by promoting the ionized state of the polymer. Being ERL and ERS cationic polymethacrylates, their mucoadhesive properties could be explained by the positive charge in the polymer structure.

In this investigation we sought to evaluate systematically the performance of ERS and ERL as mucoadhesive polymers to be suitable for the delivery of the water soluble model drug caffeine. A series of films containing increasing quantities of caffeine revealed the appearance of agglomerates and the effect of these was evaluated in terms of mucoadhesion as well as content uniformity, mechanical properties, drug release, and morphology.

3.2. MATERIALS

Eudragit RSPO and RLPO (ERS and ERL) were kindly donated by Evonik Industries (Essen, Germany). Carbopol® 974P (C974P) and Noveon® AA-1 Polycarbophil (PCP) were donated by Lubrizol Advanced Materials (Cleveland, OH). Triethyl citrate (TEC; Morflex Inc., Greensboro, NC), mucin (Spectrum Chemical, New Brunswick, NJ), and caffeine (CAF, Sigma-Aldrich, St. Louis, MO) were purchased and used as received. All other chemicals used were of analytical or reagent grade.

3.3. METHODS

3.3.1. Preparation of films

For ERS and ERL series of films, polymers were firstly dissolved in an acetone:isopropanol (4:6 ratio) solvent system and then 10% w/w TEC was added as plasticizer. Increasing quantities of caffeine were added to yield solutions containing 1, 2, 3, 4, or 5% w/w caffeine. Films made of both, ERS and ERL polymers were obtained for each concentration. These solutions were casted on PTFE plates and let to dry overnight at 40 °C to yield the final product. Films were peeled off and stored in aluminum foil sachets in a dessicator until characterization. To compare with conventional mucoadhesive materials, films containing C974P and PCP were manufactured similarly. Adequate amounts of the polymers were dissolved in ethanol and then cast in the same fashion as described above.

3.3.2. Morphology of films

To observe the ultrastructure of films, scanning electron microscopy (SEM) was performed on the surface and cross sections of films. Samples were obtained by a freeze fracture method to ensure clean-cut edges and to avoid plastic deformation (often resulting from mechanical cutting). Fragments of the surface of the film were frozen by submerging in liquid nitrogen and thus cracked by freezing. Pieces of the films were fixed on aluminum stubs by means of conductive carbon tape. A Cressington 208 HR

sputter coater (Cressington Scientific Instruments Ltd, Watford, UK) was used to coat samples with Pt/Pd to a thickness of 10–15 nm in a high vacuum evaporator. A Hitachi S-5500 field emission scanning electron microscope (SEM, Hitachi High-Technologies Corp., Tokyo, Japan) was operated for imaging of coated particles. The electron beam voltage was kept at 2-5 kV to avoid structural deformation during imagining [21].

An energy dispersive spectroscopy detector (Bruker EDS Quantax 4010) installed in the SEM was used to analyze elemental distribution and 2-dimensional mapping of selected elements. Although caffeine and both Eudragit possess the same elements, the concentration of nitrogen in caffeine is higher and was used to elucidate caffeine-rich domains in cross-sections of films.

3.3.3. Mechanical properties of films

Using a TA.XTPlus texture analyzer (Stable Micro Systems, Godalming, UK) equipped with a 5Kg load cell, stress versus strain curves were obtained and the mechanical properties of film strips were determined. Briefly, rectangular strips of 1 x 5 cm^2 were cut and 1 cm on each end was held between clamps attached to the texture analyzer, leaving a testing area of 1 x 3 cm^2 for determination of mechanical properties. The upper clamp (connected to the mobile arm of the texture analyzer) was moved upwards at a rate of 0.5 mm/sec until film failure. Stress is obtained from the force measurements obtained from the instrument divided by the cross-sectional area of the film, while strain is computed by dividing the increase in length by the initial film length.

From the plot, the tensile strength (TS) and the elongation at break (EB) are obtained from the peak stress and the maximum strain, respectively, also represented by the following equations [3]:

$$Tensile\ strength\ (TS) = \frac{Peak\ stress}{Cross - sectional\ area\ of\ film}$$

$$Elongation\ at\ break\ (EB) = \frac{Increase\ in\ length\ at\ break}{Initial\ film\ length} \times 100$$

Additionally, the elastic modulus (EM) was obtained from the initial elastic deformation region in the stress vs. strain plot [22]. Since the rate of the mobile arm was constant during the test as well as for all different experiments, direct comparison of the slope in this region can be done. To further evaluate mechanical properties three additional parameters were computed from the conventional mechanical parameters obtained from the plot as follows [23]:

$$Tensile\ strength\ to\ modulus\ ratio = \frac{TS}{EM}$$

$$Relative\ surface\ energy\ (RSE) = \frac{TS^2}{2 \times EM}$$

$$Toughness\ index\ (TI) = \frac{2}{3} \times TS \times EB$$

3.3.4. Mucoadhesion of films

Mucoadhesion tests were conducted on the texture analyzer equipped with a 5 Kg load cell. Briefly, films were held in the horizontal position and 5 µL of model mucus (a freshly made 2% w/v mucin solution) was placed on top of the film. This amount is sufficient to mimic the thickness of the average saliva thickness [24]. A 7 mm diameter stainless steel cylindrical probe was attached to the mobile arm of the texture analyzer and it was brought in contact with the film and mucin solution, held at an applied force of 50 mN for 15 seconds and then withdrawn at a 0.5 mm/second rate. Mucoadhesive force (MAF) and work of adhesion (WoA) are obtained from the peak and the area under the curve in the force versus distance profile, respectively.

3.3.5. Caffeine assay

Caffeine concentration in samples obtained above was determined by UV spectroscopy using a µQuant microplate reader (Bio-Tek Instruments, Inc, Winooski, VT). Briefly, 300 µL aliquots were added in each well in the microplate in triplicates. UV absorbance was measured at 273 nm and the concentration was calculated from a calibration curve of a stock solution of caffeine.

3.3.6. Drug content uniformity

To measure the average amount of drug loading in the films and to determine homogeneity among the cast surface, film samples were analyzed for caffeine content uniformity. Samples were cut to yield 1 x 1 cm^2 squares and allowed to release caffeine for 24 hours in 15 mL phosphate buffer pH 6.8 in an orbital shaker at 20 °C. Aliquots from these vials were analyzed for caffeine content using the UV spectroscopy method described above.

3.3.7. In vitro drug release

Dissolution tests were conducted to determine drug release profiles from Eudragit films. A small vessel USP apparatus I (basket) was used for this purpose and 150 mL phosphate buffer pH 6.8 was used as dissolution media. Film were cut into 1 x 1 cm^2 samples and dissolved into each vessel with a rotating speed of 25 rpm at 37 °C. At intervals of 0, 0.25, 0.5, 1, 2, 3, and 4 hours 1 mL samples were withdrawn and replaced with 1 mL of fresh warm media. Caffeine concentration was determined as depicted above using a UV spectroscopy method of quantification. Comparison of the release profiles was performed using the similarity factor, f2 [25].

3.3.8. Kinetic analysis of release profiles

Kinetic models were used to compare the release mechanisms from the various caffeine-containing films. The Higuchi [26], Korsmeyer-Peppas [27], and first order

kinetic models were used to fit the data and were compared on the basis of R^2 adjusted [28] and the Akaike information criterion (AIC) [29]. The evaluation of the drug transport mechanism was addressed in accordance with the Korsmeye-Peppas model.

3.3.9. Statistical analysis

All statistical analyses were performed with the software Minitab Release 14® (Minitab Inc., State college, PA). One-way ANOVAs were used for multiple comparisons and Tukey's post-hoc pairwise comparisons were performed to compare which results led to significant differences. All values are reported as the mean and standard deviation of the mean in parenthesis. For the evaluation of the kinetics models and calculation of adjusted R^2 values the software Origin® 8.0 (Northampton, MA) was used to perform the non-linear regressions for each equation.

3.4. RESULTS AND DISCUSSION

3.4.1. Morphology of films

SEM images shown in Figure 3.1 and 3.2 reveal that increasing the concentration of caffeine in both ERS and ERL films leads to an increasing appearance of agglomerates in cross sections of films obtained by freeze fracture. A survey of cross sections reveals that the use of ERS leads to a higher quantity and larger size of these agglomerates at similar concentrations of caffeine compared to those seen in ERL films. For example, ERS03 reveals a larger number of the needle-like agglomerates compared to ERL03

(Figure 3.2). In addition, ERS04 reveals the appearance of larger agglomerates possibly composed of aggregation of the needle like caffeine crystals observed at lower concentrations, while ERL04 still shows only needle like agglomerates.

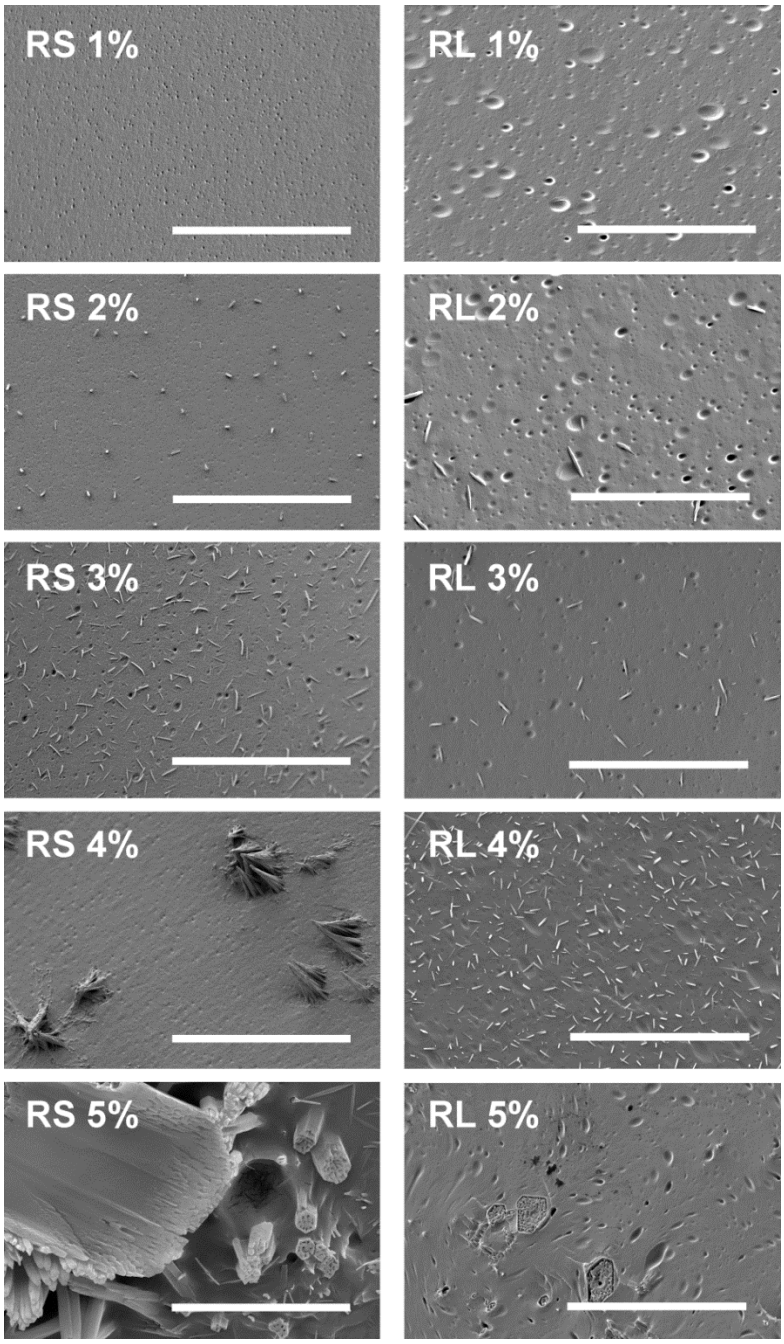

Figure 3.1. SEM images of ERS and ERL films at various concentrations of caffeine. Bar represents 30 μm.

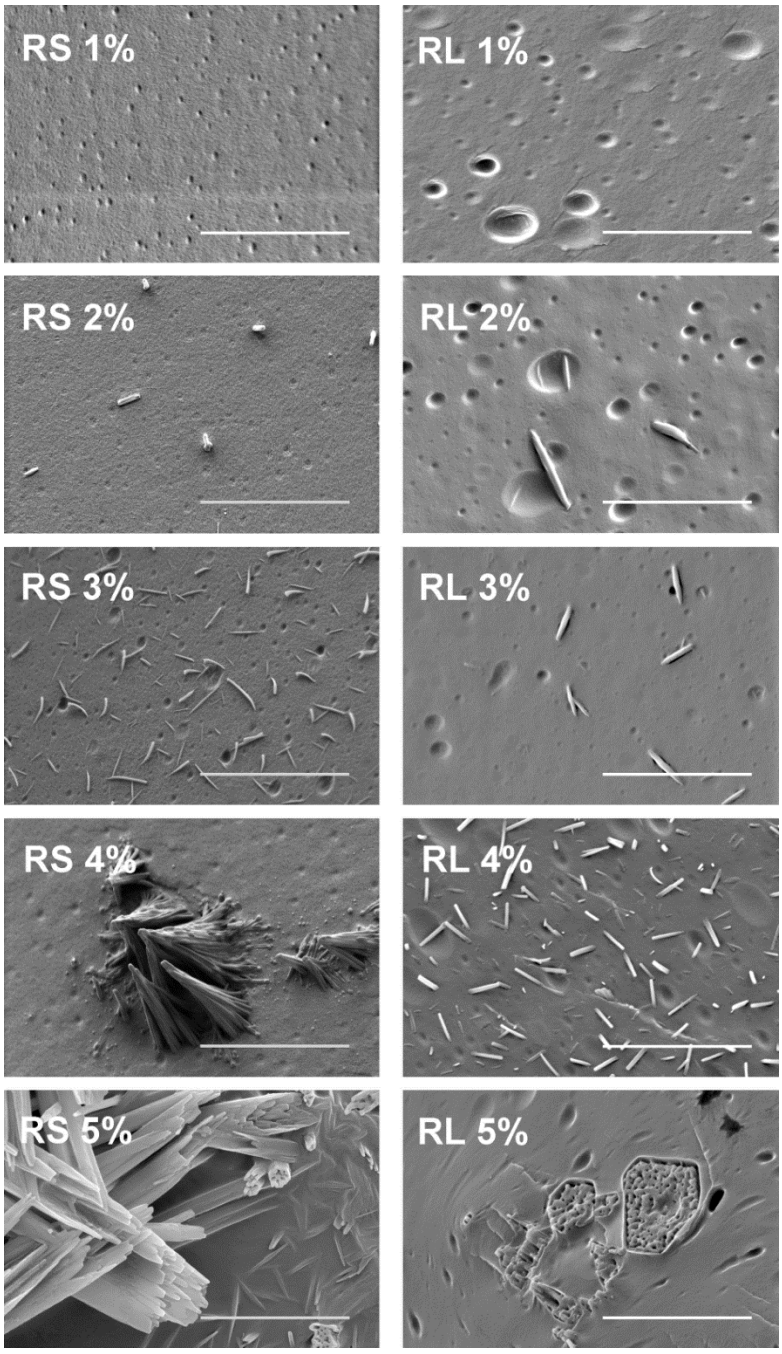

Figure 3.2. SEM images of ERS and ERL films at various concentrations of caffeine. Bar represents 10 μm.

EDS mapping of nitrogen (Figure 3.3) on SEM scan fields revealed that the agglomerates consist of caffeine and appear to have an organized crystalline structure, which is also appreciated at higher magnification micrographs obtained for formulations with higher content of caffeine (Figure 3.2). Even though, the polymer structure possess nitrogen atoms branching out of the backbone, the density of nitrogen atoms is higher in the caffeine molecule than the polymer, thus for the same time of detection of X-rays emitted from the field of view of the sample, the bulk of the signal can be attributed to caffeine [30]. The shape of the agglomerates observed in cross-sections of the films is also consistent with caffeine crystals shapes reported in the literature. It has been reported in the literature that when recrystallized from organic solvents, anhydrous caffeine crystals can adopt different space groups in a rhombohedral lattice system including but not limited to R3c and R3 [31,32]. These space groups result in hexagonal prisms, which concur with the SEM observations. The difference in the extent of caffeine recrystallization and size and number of agglomerates can be attributed to the differences in hydrophillicity elicited by both ERS and ERL [33]. Both, ERS and ERL, are pH independent and insoluble but swellable in water polymers. This is due to the quaternary ammonium groups that branch out of the polymethacrylate backbone of the polymer structure. The ammonium groups are present as salts and allow for swelling of the polymer. ERL is the more permeable polymer due to its content of about 10% of the ionic functional groups, while the content for ERS is approximately 5% [34]. Therefore, ERL

can solubilize to a higher extent than ERS, and caffeine in the polymer matrix increasingly retards the appearance of large agglomerates with increasing concentrations. A similar effect has been observed by Omari et al. (2004) where the interaction between lactic acid and ERL and ERS were compared [35]. Lactic acid-containing ERL films revealed a higher extent of interaction by differential scanning calorimetry (DSC) and nuclear magnetic resonance (NMR) studies. This effect was attributed to the higher hydrophillicity featured by ERL compared to ERS allowing for a further ionic interaction with the acid. This effect also accounted for an increase in drug permeation when release of paracetamol was studied. It was found that lactic acid clearly modified the release in ERL due to the higher extent of interaction as opposed to ERS films in which the modification of permeation was less pronounced.

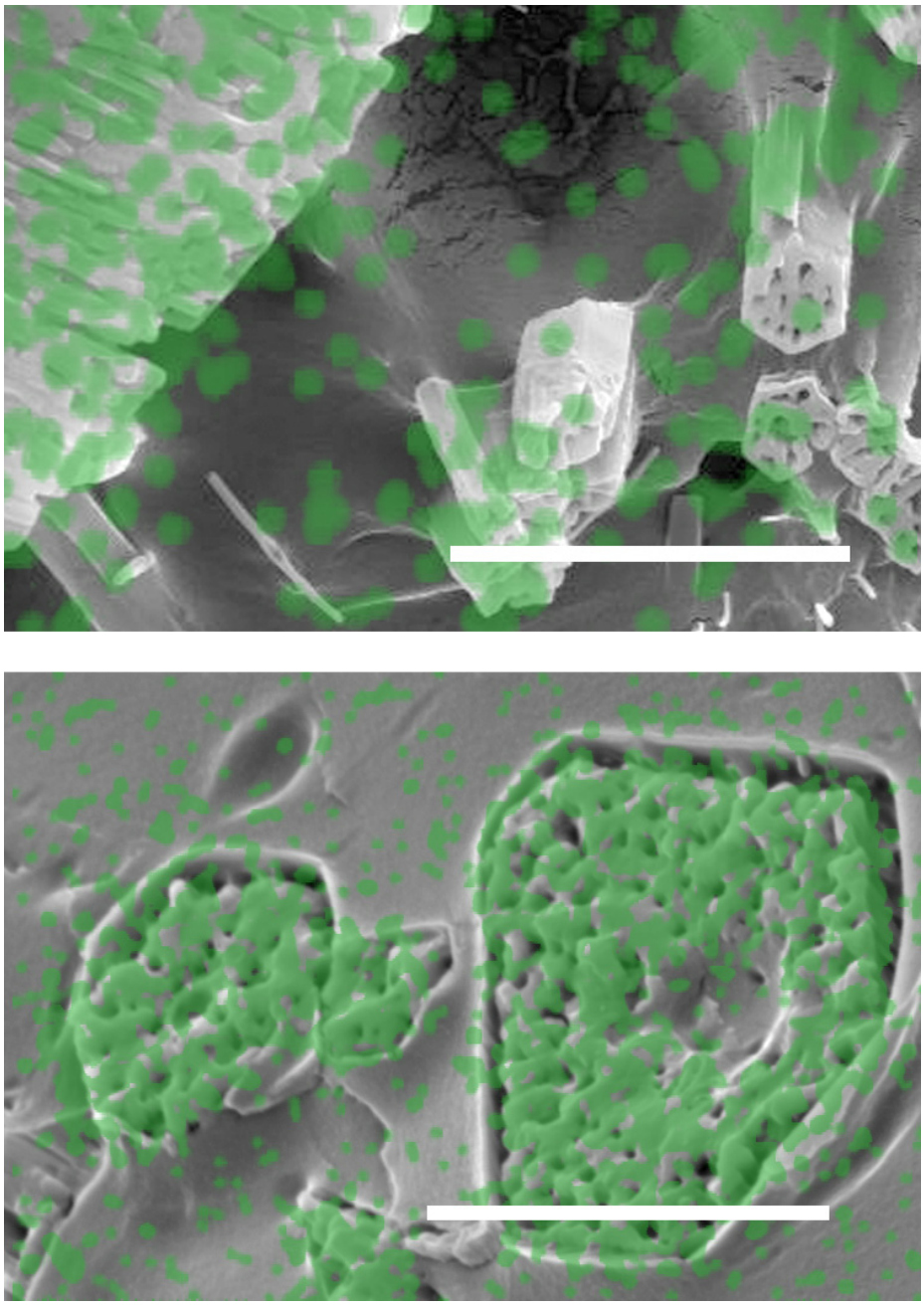

Figure 3.3. SEM images merged with EDS mapping for nitrogen (in green) showing that caffeine is highly concentrated in the crystalline agglomerates found in ERS05 and ERL05. Bar represents 10 μm.

3.4.2. Mechanical and mucoadhesive properties

The mechanical properties of a film as a solid dosage form are of great importance since they account for the ability of the film to withstand various sources of stress. First, films need to withstand the stress imposed by the manufacturing, handling, and administration [17]. Additionally, films for buccal delivery need to be able to remain in contact with the mucosa for as long as the delivery of the active is ongoing [36]. This involves mechanical stress originating from various mouth activities. Therefore, films are preferred to exhibit a relatively high TS, EB, and a low EM [36]. In addition, regarding derived mechanical parameters, a relatively high TS/EM, RSE, and TI are desired [23,35].

From stress vs. strain curves, TS, EB, and EM were obtained and the derived magnitudes of TS/EM, RSE, and TI were computed for each sample and are summarized in Table 3.1 and 3.2. TS/EM is a measure of the level of internal stress in a film. The larger its value the higher the film crack resistance. RSE is also utilized to estimate crack resistance and is approximated from the surface energy of the film. Finally, TI is an estimation of energy absorbed per unit volume of film under stress [23]. In Table 3.1, it can be evidenced that films from the ERL series have a significantly lower TS and EM, but a higher EB than each of the corresponding ERS film, indicating that ERL is a softer and more elastic material than ERS. However, when both TS and EB are taken into account as TI we can observe that the increase in EB for ERL compensates the decrease

in TS yielding tough films at all concentrations of caffeine except for ERL05. Additionally, analysis of TI also reveals that ERS04 and ERS05 are less tough films, which is not evident by a direct analysis of conventional mechanical parameters [35]. Results of TS, EB, and EM indicated a significant difference on both ERS05 and ERL05, as well as ERS04 with respect of EB. As discussed above, as concentration of caffeine increases the capacity of the polymer to dissolve the drug content reaches a saturation point allowing for recrystallization. It has been suggested in the literature that unsolubilized drug, which in our case would result in recrystallization, can physically interrupt the polymer matrix resulting in hard and brittle films [37]. This is also consistent with inspection of ERS04 micrographs in which we can observe large agglomerates, similar to those found in ERL05.

Table 3.1. Mechanical properties of formulations from ERS and ERL series. Values are represented as average and standard deviation in parenthesis.

Formulation	Tensile Strength /N/mm^2		Elongation at Break /%		Elastic Modulus /N/mm^2/%	
ERS01	5.71	(1.72)^{a}	142.19	(35.46)^{ab}	1.19	(0.46)
ERS02	3.62	(0.61)	162.40	(44.06)^{cde}	1.18	(0.04)
ERS03	4.16	(0.86)	82.88	(20.74)^{c}	1.33	(0.26)
ERS04	4.04	(0.83)	35.30	(6.24)^{ad}	1.27	(0.36)
ERS05	2.48	(0.14)^{a}	35.82	(17.99)^{be}	0.80	(0.09)
ERL01	1.51	(0.19)^{ab}	233.04	(23.85)^{a}	0.43	(0.05)^{ab}
ERL02	1.17	(0.13)^{acd}	262.21	(34.06)^{b}	0.34	(0.05)
ERL03	0.75	(0.06)^{bcef}	275.23	(35.84)^{cd}	0.24	(0.03)^{a}
ERL04	1.26	(0.17)^{e}	221.83	(30.38)^{ce}	0.43	(0.04)
ERL05	1.51	(0.14)^{df}	93.41	(9.65)^{abde}	0.63	(0.07)^{b}

a, b, c, d, e, f: Among parameters and between series of formulations, statistically significant differences are paired by the same letters ($p<0.01$).

Table 3.2. Derived mechanical parameters calculated from conventional mechanical properties derived from a Stress vs. Strain plot. Values are represented as average and standard deviation in parenthesis.

Formulation	TS:EM $\%^{-1}$	Relative Surface Energy $N/mm^2 \cdot \%$	Toughness index $N/mm^2 \cdot \%$
ERS01	4.98 (0.84)[a]	13.90 (3.23)[abcd]	515.88 (38.39)[abc]
ERS02	3.23 (0.53)[a]	6.25 (1.95)[a]	391.16 (116.40)[def]
ERS03	3.14 (0.46)	6.60 (1.97)[b]	222.07 (5.69)[adg]
ERS04	3.35 (1.14)	6.91 (3.27)[c]	96.82 (34.12)[be]
ERS05	3.16 (0.54)	3.95 (0.91)[d]	58.53 (28.48)[cfg]
ERL01	3.31 (0.65)	2.38 (0.71)	233.04 (27.10)[ab]
ERL02	3.29 (0.23)	1.80 (0.07)	204.80 (33.14)[cd]
ERL03	3.14 (0.71)	1.17 (0.37)	136.80 (8.07)[ac]
ERL04	2.94 (0.57)	1.88 (0.63)	186.94 (41.36)[e]
ERL05	2.44 (0.39)	1.86 (0.45)	94.53 (15.45)[bde]

a, b, c, d, e, f, g: Among parameters and between series of formulations, statistically significant differences are paired by the same letters ($p<0.01$).

Being ERS and ERL both water-insoluble polymers they are normally regarded in the literature as non-mucoadhesive materials [16,18]. The results observed in Figure 3.4 and 3.5 reveal that the mucoadhesive properties of ERS are very limited both in terms of MAF and WoA and comparatively always lower than their ERL counterparts. Only when caffeine is in a solid solution with the polymer (ERS01) a significantly higher MAF of 65.04±6.44 mN is found compared to other ERS formulations ($p<0.05$), although in

comparison with the more hydrophilic ERL MAF is much lower (211.11±24.29 mN for ERL01). Conversely, ERL is highly mucoadhesive under the test conditions utilized here. This is not surprising when we consider that even though the polymers are water-insoluble they are swellable in water due to the presence of the quaternary nitrogen groups. The ability of hydrophilic polymers to swell in water is a common characteristic in materials generally recognized as mucoadhesives and is consistent with various of the theories of mucoadhesion [38–40]. In saliva, the most relevant component to mucoadhesive interactions is mucin which is the main component in our saliva model. Mucins are composed of a protein core and carbohydrate side chains, which are responsible for the non-covalent bonding that occurs when a mucoadhesive material is brought in contact with mucosa [41,42]. According to the diffusion theory [38] interpenetration and entanglement between polymer chains (mucin and mucoadhesive material) is believed to be the main reason for mucoadhesive bonding. Control experiments utilizing only the mucus model and the stainless steel probe revealed very little contribution of the mucus-steel interface to the measured force (MAF equals 12.96±1.95 mN and WoA equals 2.70±0.28 μJ). Use of the same experimental set up revealed that the extent of mucoadhesion found with ERL is comparable to that of typical mucoadhesive materials, namely C974P and PCP (Figure 3.5) [19,43]. Particularly, the formulation exhibiting the highest MAF (ERL01) is about 30% significantly lower than both C974P and PCP (211.1 vs. 329.7 and 301.1 mN, respectively). It was found however

that the WoA was about 80% significantly higher than conventional mucoadhesive materials (118.9 vs. 23.9 and 17.4 μJ), demonstrating that a highly swellable polymer, such as ERL, regardless of being water-insoluble, can elicit strong mucoadhesiveness based on its capacity for entanglement. The various films in the ERL series exhibit high WoA and high MAF when the drug is solubilized in the polymer or small micron size agglomerates are found (ERL01-ERL04); however, the highest concentration of caffeine that renders large recrystallized agglomerates results in a significant decrease of both mucoadhesive variables. This is also in correlation with findings discussed above in terms of morphology and mechanical properties.

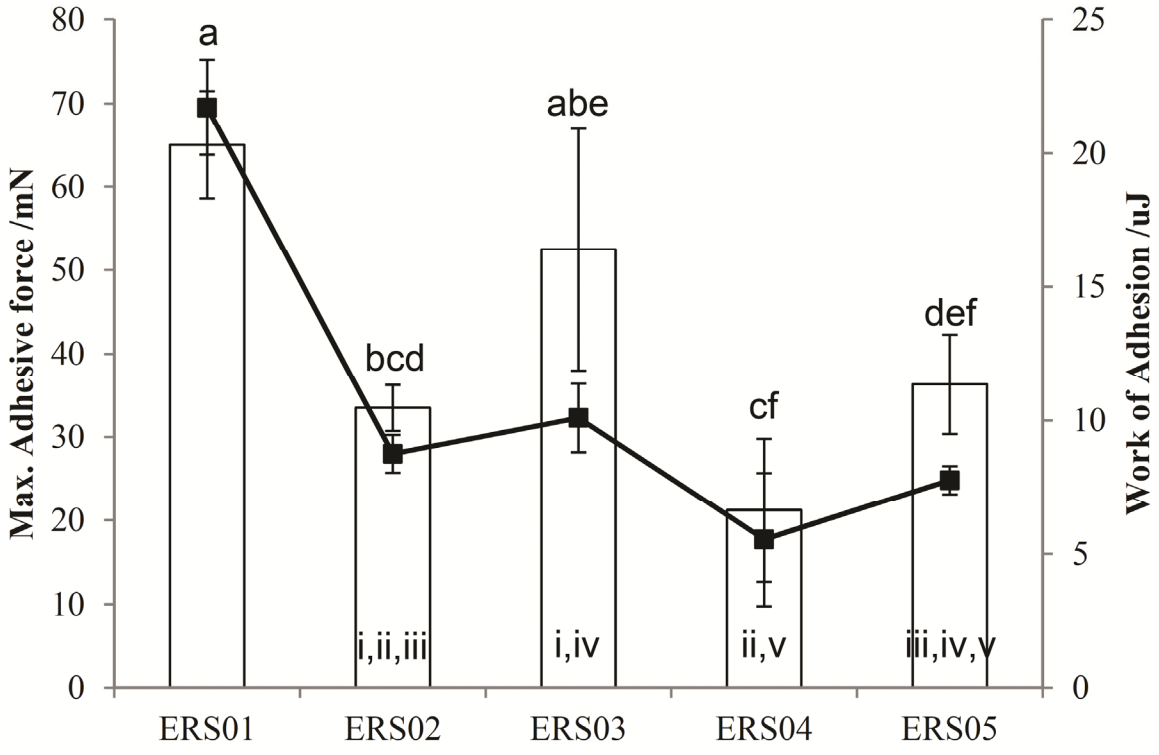

Figure 3.4. Mucoadhesive properties of ERS films: Maximum Adhesive Force (□) MAF, with non-significant differences indicated in pairs of letters (a-f); and Work of Adhesion (■) WoA, with non-significant differences indicated in pairs of roman numerals (i-v).

The consistent decrease of mucoadhesive and mechanical properties as concentration of caffeine increased led us to investigate the existence of a correlation between the two. After a linear regression analysis, the data shows a strong positive correlation between EB and MAF regardless of the polymer type (r = 0.9). Although further investigation would be required on this topic, particularly isolating variables to allow for a more accurate evaluation, there could be a connection between elasticity of films and measurement of mucoadhesion by the method utilized here. This could be

explained as follows: stiff films will not be able to deform enough to allow for a prolonged contact during detachment; therefore, resulting in less force needed to break the detachment. More ductile films will be able to support the mucoadhesive bond for longer and will require larger inputs of energy for detachment. This is further corroborated by a strong correlation between EB and WoA for ERL ($r = 0.9$) indicating the possibility for such interaction between mechanical and mucoadhesive properties for films as dosage forms.

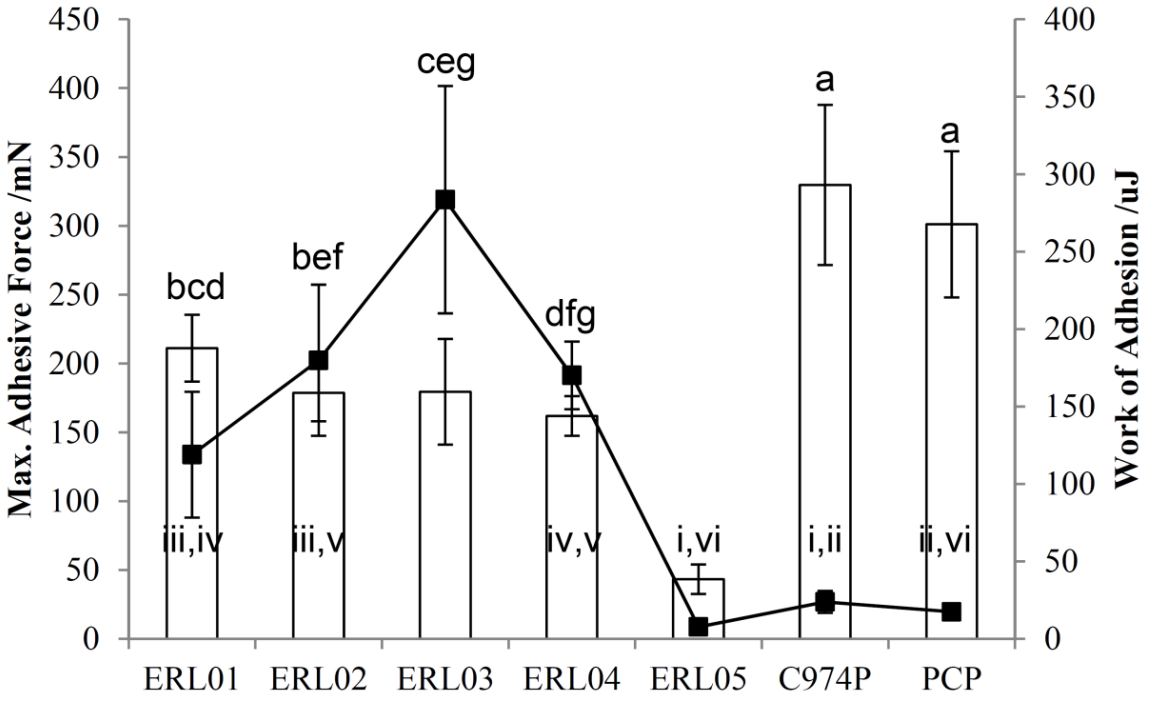

Figure 3.5. Mucoadhesive properties of ERL films and C974P and PCP as conventional mucoadhesive polymers: Maximum Adhesive Force (□) MAF, with non-significant differences indicated in pairs of letters (a-g); and Work of Adhesion (■) WoA, with non-significant differences indicated in pairs of roman numerals (i-vi).

3.4.3. Drug content uniformity, drug release, and kinetics

The increase of caffeine in films was correlated with an increase in heterogeneity of drug distribution in the casting surface of films as can be depicted in Figure 3.6. Up to a content of 2% caffeine, films exhibit very high drug content uniformity (relative standard deviation ≤1.7%), while at higher concentrations heterogeneity is evident. This is in accordance with the ultrastructure of films obtained by SEM. Both ERS03 and ERL03 present with more numerous agglomerates of caffeine which are not uniformly

distributed when panning under the microscope is performed in larger areas (Figure 3.1). A similar situation is found at higher concentrations of caffeine in addition to the appearance of larger recrystallized agglomerates of caffeine, which contributes to the loss of homogeneity. As hinted above, the extent of the drying times has been acknowledged in the literature as one factor that will allow for particle agglomeration [9,10,44]. Strategies such as the addition of gelling and viscosing agents, increasing the rate of drying, and/or casting in unitary wells have all been addressed in the literature as means to increase uniformity and could allow us to improve uniformity at higher concentrations of caffeine.

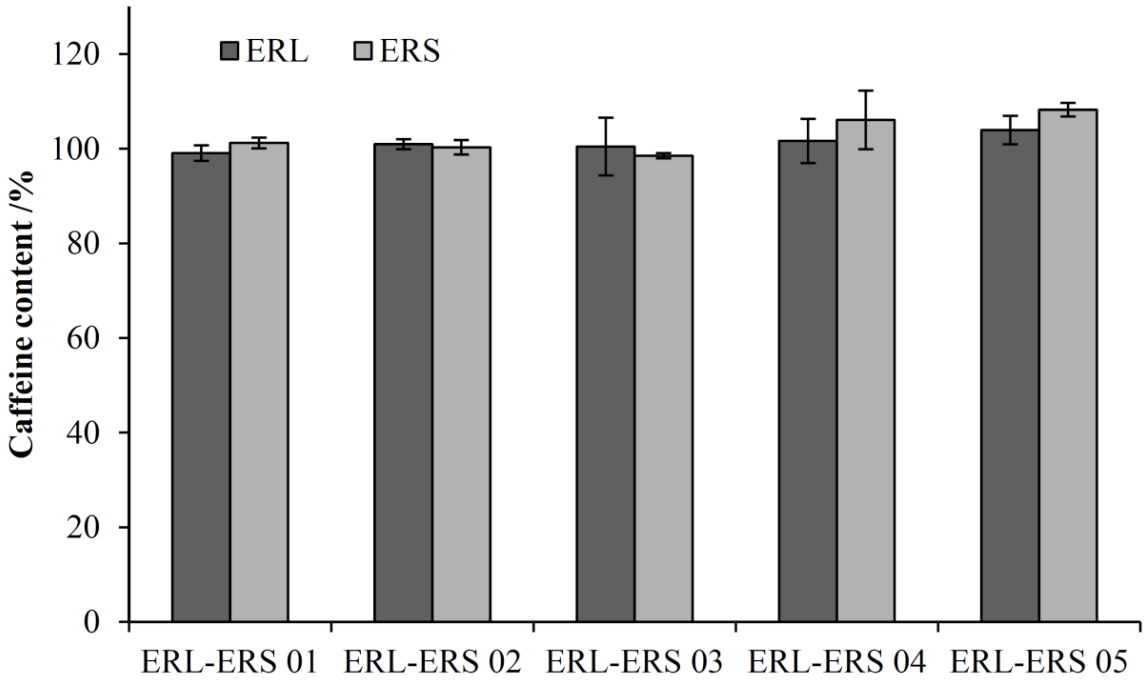

Figure 3.6. Caffeine content uniformity for ERL and ERS series. Darker grey columns represent the ERL series, while the lighter grey columns represent the ERS series. Values (mean ± standard deviation, n=4-6) are reported as percentages of the theoretical amount of caffeine in each sample studied. Differences among all ten formulations are not statistically significant ($p>0.05$).

Due to the high permeability to water of ERL no differences could be evidenced in release profiles and almost complete release of the drug regardless of the concentration was achieved after 30 minutes (Figure 3.7). Using the similarity factor, f2 [25], it was determined that all of the release profiles were similar (f2 >50%, Table 3.3). Conversely, all the release profiles for the ERS series, except between ERS02 and ERS04, were different between each other per f2 (data not shown). ERS behaved as expected from the literature allowing for controlled release of caffeine at every concentration studied as

depicted in Figure 3.8 [45]. As the concentration of caffeine increased the rate of drug release increased as well. This can be attributed to a faster penetration of the water front through the polymer by dissolving agglomerates rather than displacing caffeine molecules from the polymer matrix (ERS01) [46].

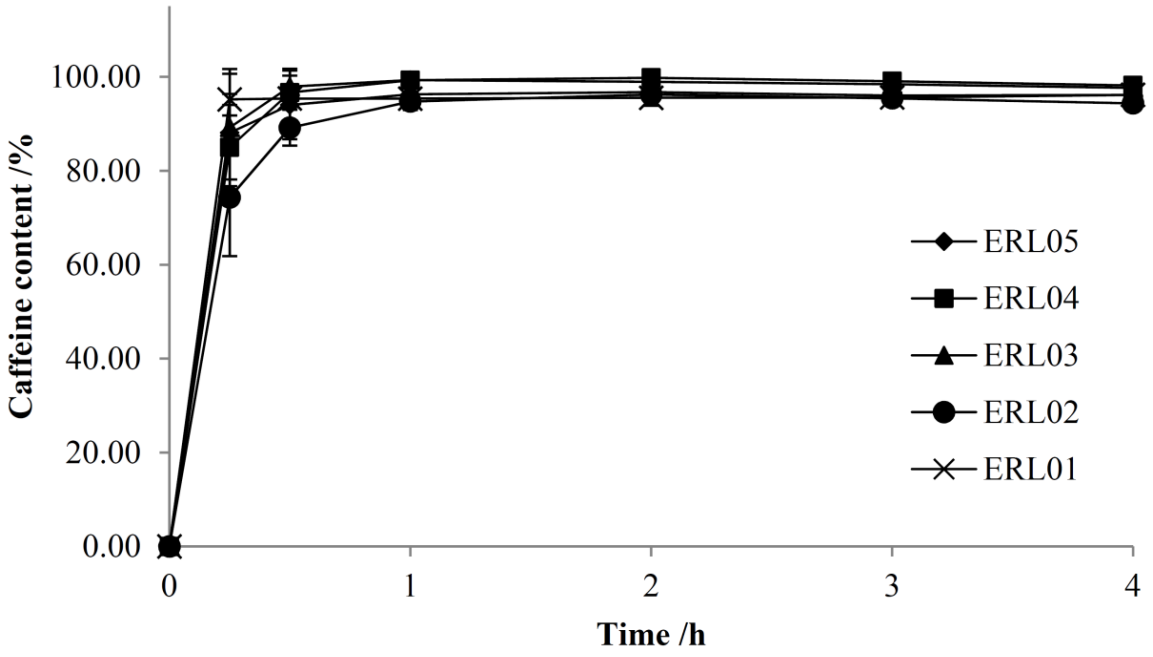

Figure 3.7. Drug release profiles for ERL series in phosphate buffer pH 6.8 at 37 °C, showing (♦) ERL05, (■) ERL04, (▲) ERL03, (●) ERL02, and (□) ERL01. Values are presented as mean ± standard deviation, n=6.

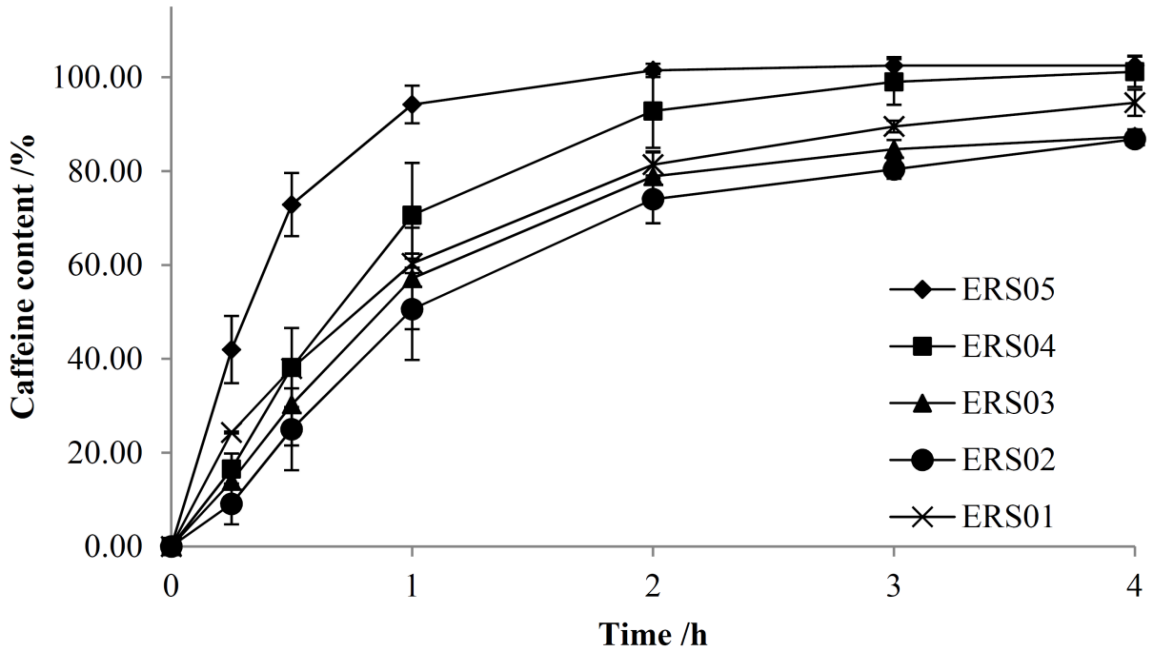

Figure 3.8. Drug release profiles for ERS series in phosphate buffer pH 6.8 at 37 °C, showing (♦) ERS05, (■) ERS04, (▲) ERS03, (●) ERS02, and (□) ERS01. Values are presented as mean ± standard deviation, n=6.

Table 3.3. Differences among formulations of ERS and ERL series based on the similarity factor, f2. Release profiles are similar if f2 ≥50.

f2	ERS05	ERS04	ERS03	ERS02	ERS01
ERS01	21.0	41.2	48.0	37.7	--
ERS02	12.9	25.0	54.3	--	
ERS03	16.1	30.4	--		
ERS04	26.8	--			
ERS05	--				
f2	ERL05	ERL04	ERL03	ERL02	ERL01
ERL01	73.5	56.9	61.9	51.1	--
ERL02	58.6	52.0	50.3	--	
ERL03	66.9	81.2	--		
ERL04	64.0	--			
ERL05	--				

In Table 3.4, it is interesting to note that as the concentration of caffeine increases the release mechanism model that best explains the data (by comparison of the adjusted R^2 and AIC) changes from a diffusion controlled mechanism (Korsmeyer-Peppas kinetics model) to a first order mass balance (first order model). In the Kormeyer-Peppas release kinetics model, n is the release exponent, and is an indicative of the drug release mechanism [27]. In the particular case of n equal to 0.5 the drug release mechanism is purely Fickian diffusion (the particular solution that constitutes the Higuchi model equation). When n equals 1 the equation describes a zero order release mechanism, and

the region ranging from 0.5 <n <1 represents the so-called anomalous transport. The first order kinetics applies to dosage forms that normally contain water-soluble drugs and porous polymer matrices. In said systems, drug release is proportional to the amount of drug remaining inside; therefore, the rate of drug release decreases with time. In accordance with the Korsmeyer-Peppas model, all except for ERS02 follow an anomalous transport implying that drug is transported by a combination of diffusion and case-II transport, characteristic of systems swelling in water (Table 3.4). ERS02 follows what has been described as a super case-II transport mechanism [28] and has been attributed to the result of an increased plasticization at the relaxing boundary (gel layer) [47,48].

Table 3.4. Model parameters, adjusted R^2, and Akaike information criteria (AIC) values for ERS series.

Formulation	Korsmeyer-Peppas $Q = k \times t^n$				Higuchi $Q = k \times t^{0.5}$			First order $Q = k \times (1 - e^{-nt})$			
	k	n	Adj R^2	AIC	k	Adj R^2	AIC	K	n	Adj R^2	AIC
ERS01	0.637	0.660	0.9998	10.09	0.604	0.9912	10.15	0.876	1.288	0.9966	9.29
ERS02	0.586	1.119	0.9876	10.77	0.540	0.9391	37.07	1.090	0.693	0.9849	29.36
ERS03	0.657	0.973	0.9957	6.86	0.602	0.9409	29.45	5.993	0.116	0.9986	6.10
ERS04	0.703	0.974	0.9896	12.15	0.625	0.9421	31.10	5.854	0.127	0.9964	11.66
ERS05	0.940	0.528	0.9125	20.40	0.929	0.9873	18.61	1.055	2.106	0.9964	13.92

3.5. CONCLUSION

In contrast with what has been previously reported in the literature, we have found that ERS and more noticeably ERL have substantial mucoadhesive properties. This was further corroborated by direct comparison with materials typically regarded in the literature as being good adhesives, namely Carbopol 974P and Polycarbophil. In accordance with the diffusion theory of mucoadhesion, this was attributed to the swelling capacity of these polymers due to the presence of quaternary ammonium groups that increase hydrophilicity. Additionally, we have found through direct observations under the microscope that increasing concentrations of caffeine in ERS and ERL matrices yielded recrystallized agglomerates. These agglomerates increase in number and size due to solubility saturation as the concentration of caffeine was increased, which translated not only in a detriment of the mucoadhesive properties, but also in reduced mechanical and uniformity properties in the film. Finally, it was shown that the presence of these agglomerates changes the release kinetics of the films from a diffusion controlled mechanism to a first order mass balance with the increased caffeine loading. Thus, size of particulate material in films was found to preferably be below one micron. This determined the need for submicron or nanoparticles to carry the macromolecules investigated in this dissertation. The method of manufacture of nanoparticles and the addition of nanoparticles on films for buccal delivery are depicted in the following chapters.

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4. A Design of Experiments to Optimize a New Manufacturing Process for High Activity Protein-Containing Submicron Particles[3]

ABSTRACT

A novel method for the manufacture of protein/peptide-containing submicron particles was developed in an attempt to provide particles with increased activity while using high energy input technologies. The method consists of antisolvent co-precipitation from an aqueous solution containing both an amino acid core material (e.g. D,L-valine), and either bovine serum albumin (BSA) or lysozyme (Lys) as model proteins. The aqueous solution was added to the organic phase by means of a nebulizer to increase the total surface area of interaction for the precipitation process. Sonication proved to be an effective method to produce small particle sizes while maintaining high activity of Lys. The use of a polysorbate or sorbitan ester derivatives as stabilizers proved to be necessary to yield submicron particles. Particles with very high yields (approximately 100%) and very high activity after manufacture (approximately 100%) could be obtained. A particle size of 439.0 nm, with a yield of 48.8% and with final remaining activity of 98.7% was obtained. By studying various factors using a design of experiments strategy (DoE) we were able to establish the critical controlling factors for this new method of manufacture.

[3] Significant portions of this chapter were taken from: J.O. Morales, G.M. Joks, A. Lamprecht, A.C. Ross, J.T. McConville, A design of experiments to optimize a new manufacturing process for high activity protein-containing submicron particles, Drug Development and Industrial Pharmacy. (2012) *In Press*.

4.1. INTRODUCTION

Recent increases in the number of products under review by the FDA or undergoing late phase clinical trials demonstrates the fact that protein and peptide therapeutics is a rapidly growing field in the pharmaceutical industry [1]. The development of delivery strategies for proteins and peptides has generally been limited to intravenous administration with very little success for other routes of administration; this is despite a plethora of articles in the literature supporting the fact that there is a need for alternative routes of delivery for protein and peptides. Within the literature, the most prominent alternative route of administration is by oral delivery [2–4]. However, the many limitations for gastrointestinal tract delivery has led to the exploration of other routes of delivery [5,6], such as pulmonary [7,8], nasal [9], transdermal [10], and buccal [11] routes. Regardless of the route of delivery, protein-containing submicron-/nano-particulate systems have been continuously investigated as an approach to overcome the various limitations imposed by different delivery routes [1,12].

An advantage associated with the development of submicron or sub-/nano-particulate systems is the increased amount of active solids that can be incorporated into smaller particles. Additionally, intravenous administration of smaller particles leads to faster dissolution in the blood increasing the potential to reach target organs [13]. However, one concern in the development of methods of manufacture for proteins or peptides is their labile nature. Therefore, the activity maintained following manufacture is a critical factor for consideration during the developmental stage. For example, it is known in the literature that methods where there is an interaction between proteins/peptides and organic solvents could decrease the activity due to denaturation

[14,15]. Nonetheless, recent advances in enzyme immobilization have opened the potential for antisolvent co-precipitation as a method of manufacture for protein/peptide particulate systems with similar or increased activity, when compared to similar lyophilized products [16,17]. The systems reported to date though, only describe the manufacture of particles in the range of 5-10 μm. The goal of this investigation was to adapt an antisolvent co-precipitation method for the manufacture of protein/peptide submicron particles.

Generally the antisolvent precipitation process consists of solubilizing the molecule of interest in a suitable solvent and precipitation is then triggered by adding this solution to a miscible antisolvent. In the method described here, an additional co-precipitant acts as core seed for the final precipitated particle. The co-precipitant is included in the aqueous solution containing the protein/peptide. An improvement in stability can occur following precipitation, by immobilizing the protein molecule in its native form [18,19]. An aqueous solution containing the protein/peptide and seed co-precipitant is initially prepared and admixed with an organic water-miscible solvent (e.g. 2-propanol), which can optionally contain a surfactant. Figure 4.1 depicts the general process involved in our presented antisolvent co-precipitation system. This work describes the development of the method of manufacture of submicron particles, and an investigation of the main variables controlling the process.

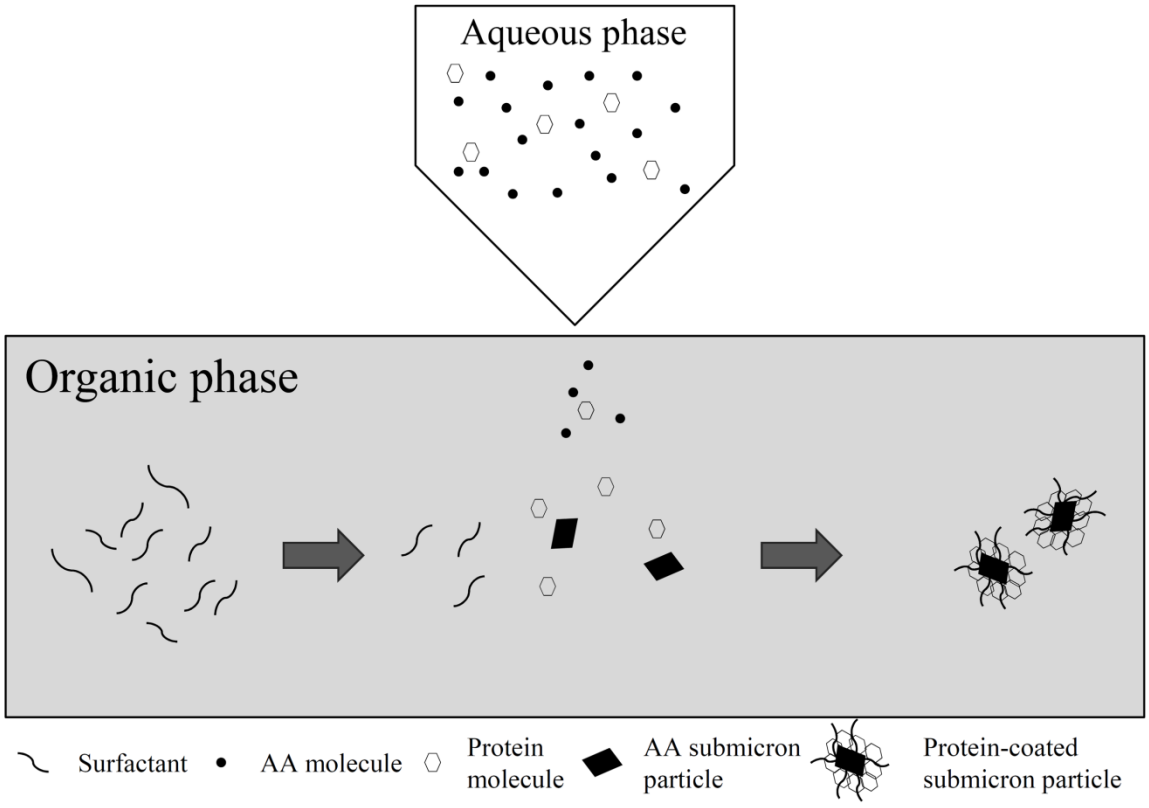

Figure 4.1. Antisolvent co-precipitation process comprising the amino acid (AA) co-precipitant and the protein molecule in the aqueous phase. The surfactant contained in the antisolvent (IPA) dehydrates the water soluble molecules yielding the submicron protein-loaded particles.

4.2. MATERIALS

Bovine serum albumin (BSA), D,L-valine (Val), L-valine, glutamine and lysozyme (Lys), were obtained from Sigma-Aldrich (St. Louis, MO). Polysorbate 80 (Tw80), sorbitan monostearate (Sp60), and sorbitan monooleate (Sp80), were obtained from Spectrum Chemical (New Brunswick, NJ). Deionized water was obtained in house

and 2-propanol (IPA) was obtained from Fisher Scientific (Fair Lawn, NJ). All other chemicals used were of analytical or reagent grade.

4.3. METHODS

4.3.1. Particle manufacturing process

The manufacturing method used was based in an antisolvent co-precipitation approach. As an overview, aqueous solutions containing the model protein and the core material (an amino acid) are prepared with the desired concentration of both ingredients. This solution is then added to the antisolvent organic phase that is completely miscible with the aqueous phase but in which there is no solubility for either the model protein or the core forming amino acid. Unless otherwise noted, the organic phase used in these studies consisted of IPA containing either Sp60 or Sp80 as surfactants. The addition of the aqueous phase was performed by spray nebulization using an Aeroneb Pro® vibrating mesh nebulizer (Aerogen, Dangan, Ireland). Depending on the formulation, either 1 mL or 5 mL was nebulized to dryness at a set rate. The total nebulization time ranged from 5–13 minutes depending on the fill volume of the nebulizer reservoir. During, and after addition of the aqueous phase via nebulization, mixing was provided by means of a Branson Sonifier 450 probe sonicator (Branson Ultrasonics, Danbury, CT). Figure 4.2 depicts the apparatus configuration that allowed for the manufacture of submicron particles by antisolvent precipitation.

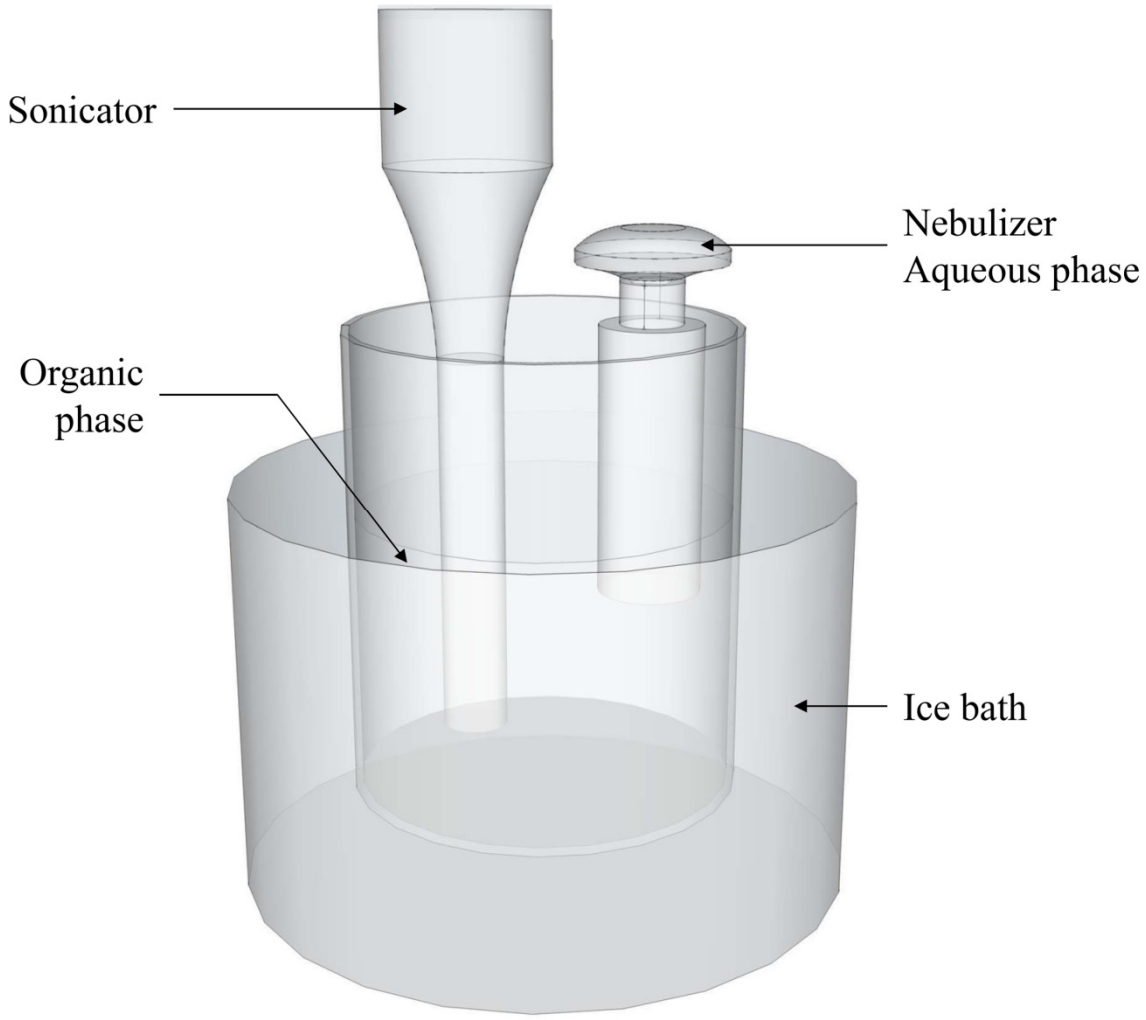

Figure 4.2. Diagram of the setup for antisolvent precipitation utilized in this investigation to yield protein-loaded submicron particles.

4.3.2. Determination of particle size

Particle sizing was performed either by using laser diffraction (LD) or dynamic light scattering (DLS) depending on the estimated particle size range after manufacture.

Laser diffraction studies were performed using a Sympatec Helos system (Sympatec Inc, Pennington, NJ). Approximately 10 mL of slurry was added to a 1 cm

path length cuvette for particle size determination (n = 4). Dilution with IPA was necessary to obtain a laser obscuration of 10-15% prior to analysis. From the particle size distributions D50 was calculated, corresponding to the diameter at which the cumulative sample volume was under 50%. Refractive indices of 1.590 and 1.378 were used for the precipitated particles and IPA respectively. To characterize polydispersity in these studies the particle size span was calculated as depicted in Equation 1

$$Span = \frac{(D90 - D10)}{D50} \tag{1}$$

Dynamic light scattering studies were conducted using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcester, UK). Approximately 1 mL of slurry, diluted with IPA when needed, was added into 1 cm path length polystyrene cuvettes for analysis of particle size. A total of 3 determinations of 15–18 runs each were conducted using the instrument. Particle size was characterized by the Z-average. The same refractive indices were used for DLS measurements. After measurement a polydispersity index (PdI) was computed by the software and it was utilized to rank slurries based on how broad the distribution was.

4.3.3. Zeta potential analysis

Zeta potentials of slurries were obtained by laser Doppler micro-electrophoresis using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Worcester, UK). Approximately 1 mL of slurry, diluted with IPA when needed, was added to a polycarbonate capillary cell for determination of zeta potential. A total of 3

determinations of 20–30 runs each were conducted to obtain the average zeta potential of the slurries.

4.3.4. Morphology of particles

A Hitachi S-5500 field emission scanning electron microscope (SEM, Hitachi High-Technologies Corp., Tokyo, Japan) was used for imaging coated particles. Particles were separated by centrifugation at 12,000 rpm then dried overnight at room temperature with an air current. Samples were mounted onto aluminum stubs using conductive carbon tape for coating. Coating was performed with a 208 HR Cressington sputter coater (Cressington Scientific Instruments Ltd, Watford, UK) with Pt/Pd to a thickness of 10–15 nm in a high vacuum evaporator. To avoid structural deformation during imaging, the electron beam voltage was kept at 2–5 kV.

4.3.5. Lysozyme yield by RP-HPLC

Lysozyme content after manufacture was determined by RP-HPLC. A known weight of dry solids was dissolved in sufficient pH 7.0 phosphate buffer, and a 500 μL aliquot was filtered and stored in vials for HPLC quantification of lysozyme. Chromatography was performed using a Zorbax 300SB® C18 Rapid Resolution column (3.5 μm, 4.6 mm ID x 150 mm length) (Agilent Technologies, Santa Clara, CA).The mobile phase consisted of two solvents with different polarities: solvent A consisted of water and with 5% v/v acetonitrile and 0.1% v/v trifluoroacetic acid, while solvent B consisted of acetonitrile, 5% v/v water, and 0.085% v/v trifluoroacetic acid. The mobile phase consisted initially of 10% v/v solvent B with a solvent gradient of 60% v/v solvent

B in 16 minutes. The flow rate was set to 1 mL/min and temperature remained constant at 25 °C. The injection volume was 50 µL and the UV detector was set at 215 nm.

4.3.6. Lysozyme activity

The enzymatic activity of lysozyme after manufacture of particles was determined turbidimetrically based on the method reported previously [20]. Activity is correlated with a decrease in absorbance at 450 nm of solutions containing *Micrococcus lysodeikticus* due to the lytic activity of lysozyme on the cell walls. A cell suspension of 0.3 mg/mL (0.9 mL) was mixed with a stock lysozyme solution containing 1 mg/mL (0.1 mL) to determine the maximum lytic effect. After separation and drying of particles, the solid was dissolved in a pH 6.2 phosphate buffer to a concentration of 1 mg/mL. Following the same procedure, sample solutions were assayed against a M. lysodeikticus suspension and absorbance was measured at 450 nm. Percentage activity was calculated by normalizing against the absorbance measured for a Lys stock solution with a known 100% activity.

4.3.7. Statistical analysis

All statistical analyses were performed with the software Minitab Release 14® (Minitab Inc., State college, PA). One-way ANOVAs were used for multiple comparisons and Tukey's post hoc pairwise comparisons were performed to compare which results led to individual significant differences. The design of experiments (DoE) and subsequent statistical analyses were also conducted using Minitab Release 14®. All values are reported as the mean and standard deviation of the mean shown in parenthesis.

4.4. RESULTS AND DISCUSSION

4.4.1. The influence of the antisolvent, core material, and the mechanism of addition of aqueous

Our antisolvent co-precipitation system as stated above is composed of a water miscible organic solvent as the antisolvent and an aqueous phase containing both the protein of interest and the core material. In our studies the selected amino acid core material demonstrated chemical compatibility with proteins. Table 4.1 summarizes the preliminary conditions that were studied using a near saturation solution of amino acid (61.2 mg/mL for Val) loaded with 10% w/w BSA as the aqueous phase. Neither acetone nor ethanol were suitable solvents to trigger the precipitation of Val, which was attributed to a higher solubility in these solvents. Both ethanol and acetone have higher polarity indices than IPA, which allow them to solubilize better the water-soluble molecules resulting in an impediment in supersaturation, ultimately resulting in the absence of precipitates. The use of IPA only resulted in smaller particle sizes when it was dehydrated. The presence of water in the organic phase makes the extraction of water from the added aqueous phase slower, which increases the mixing time, yielding larger particles during the antisolvent co-precipitation process.

Table 4.1. Study of the influence of antisolvent, core material, and style of addition of the aqueous phase in the process over particle size (D50 and span determined by LD). Results are represented as the mean and standard deviation in parenthesis. Unless specified otherwise, all pairwise comparisons are significantly different with a $p \leq 0.01$

Conditions	D50 /μm	Span
Antisolvent: IPA/water 95/5	11.04 (0.30)	1.08 (0.03)
Antisolvent: Acetone	unable to trigger precipitation	
Antisolvent: Ethanol	unable to trigger precipitation	
Core material: Val	4.98 (0.09)	1.58 (0.01)[b,c]
Core material: L-valine	17.18 (0.14)	1.75 (0.04)[a,c]
Core material: Glutamine	very large precipitates	
Aqueous added with needle	8.11 (0.27)	15.52 (0.41)
Aqueous added with nebulizer	3.04 (0.02)	1.70 (0.08)[a,b]

a, b, c: pairs of letters represent averages that do not exhibit statically significant differences with $p>0.5$.

In the antisolvent co-precipitation process, the co-precipitant needs to be water soluble and compatible with the molecules of interest (in this work BSA or Lys). Salts [21], sugar, or amino acids [22] have been proposed and investigated as core materials. Val in particular has been shown to yield enzyme-coated microcrystals [23]. We have found that among a variety of amino acids, Val provides the best characteristics as a precipitant yielding small and narrow size distributions for BSA (Table 1). Glutamine precipitated into very large, immediately sedimenting flakes, whereas L-tyrosine, L-isoleucine, L-tryptophan, N-acetyl-L-tyrosine, and L-leucine did not elicit particle formation (data not shown). It is interesting to note that L-valine yielded larger particles

than those obtained by the use of the racemic mixture. The difference in solubility (58.5 and 68.0 mg/mL for L-val and Val respectively) translated into different rates of precipitation. Since L-val has a lower solubility, the degree of supersaturation is reached faster. The nucleation rate is faster for L-val; however, the condensation and coagulation rates are also favored which results in wider particle size distribution.

During the development of the manufacturing process in this current study, the addition of the aqueous solution was performed using a syringe pump to control the rate of delivery of aqueous solution in a drop-wise fashion [24]. To explore the influence of the total surface area of interaction between the aqueous and the organic phase we analyzed the use of a nebulizer to provide droplets in the size range of 1 – 5 μm. As can be seen in Table 1, a significant decrease in D50 was observed when nebulization was utilized as a means to add the aqueous in the organic phase (3.04 vs 8.11 μm). In addition, using the nebulizer provided very narrow polydispersity reflected in the span of the size distribution. An increase in surface area by decreasing the size of the aqueous droplets that can come in contact with the organic phase is akin to an increasing Reynolds number (Re), [25] whereby there is a substantial decrease in the mixing and dehydration times. These results led us to conclude that a high surface area at the aqueous/organic interface was needed to approach smaller particle sizes with our manufacturing process.

4.4.2. Manufacture of Lys submicron particles

In previous investigations we compared the mixing methods of magnetic stirring, homogenization, and sonication (data no shown). Sonication was found to be the most effective in providing high energy mixing and small particle sizes [24,26]. During sonication, ultrasound-driven mechanical vibrations generate cavitation, where

rarefaction and compression cycles during sonication create vapor bubbles which burst after achieving a critical size. This violent cavitation creates high energy turbulence which, in addition to high speed fluid jets, is responsible in providing extremely high mixing energy to the system. This high energy mixing is equivalent to an overall reduction in the required mixing time. According to the Damkohler number (Da) reducing the mixing time (τ_{mix}) can ultimately result in faster rates of nucleation by supersaturation, this is portrayed in Equation 2:

$$Da = \frac{\tau_{mix}}{\tau_{precip}} \tag{2}$$

Where τ_{precip} is precipitation time, which is composed of τ_{cond} and τ_{coag} (condensation and coagulation times respectively) [27]. As the energy for mixing is increased, the mixing time is decreased, favoring the rate of nucleation.

Studies performed with the combination of probe sonication, the aqueous phase added by spray nebulization, and the use of a surfactant in the organic phase revealed that submicron particle sizes could be achieved for Lys-loaded particles and for Val only control particles. As can be seen in Figure 4.3, particles obtained by the antisolvent co-precipitation process conform to the typical flake-like shape of valine crystals loaded with Lys or BSA. By SEM observation, the absence of individual aggregates of different shape and the consistent flake-like shape of the particles, with and without protein, leads us to believe that the protein precipitates on the surface of the particles. This finding is similar to that described in earlier antisolvent co-precipitation studies. Kreiner et al.

(2005) used confocal laser scanning fluorescence microscopy to demonstrate that DNA was distributed on the surface of crystals by observing higher fluorescence intensity at the microcrystal edges [28]. Similarly, subtilisin Carlsberg-loaded microcrystals have been found to have a fairly uniform layer of the enzyme on the surface of the crystals by using atomic force microscope and tapping mode [23].

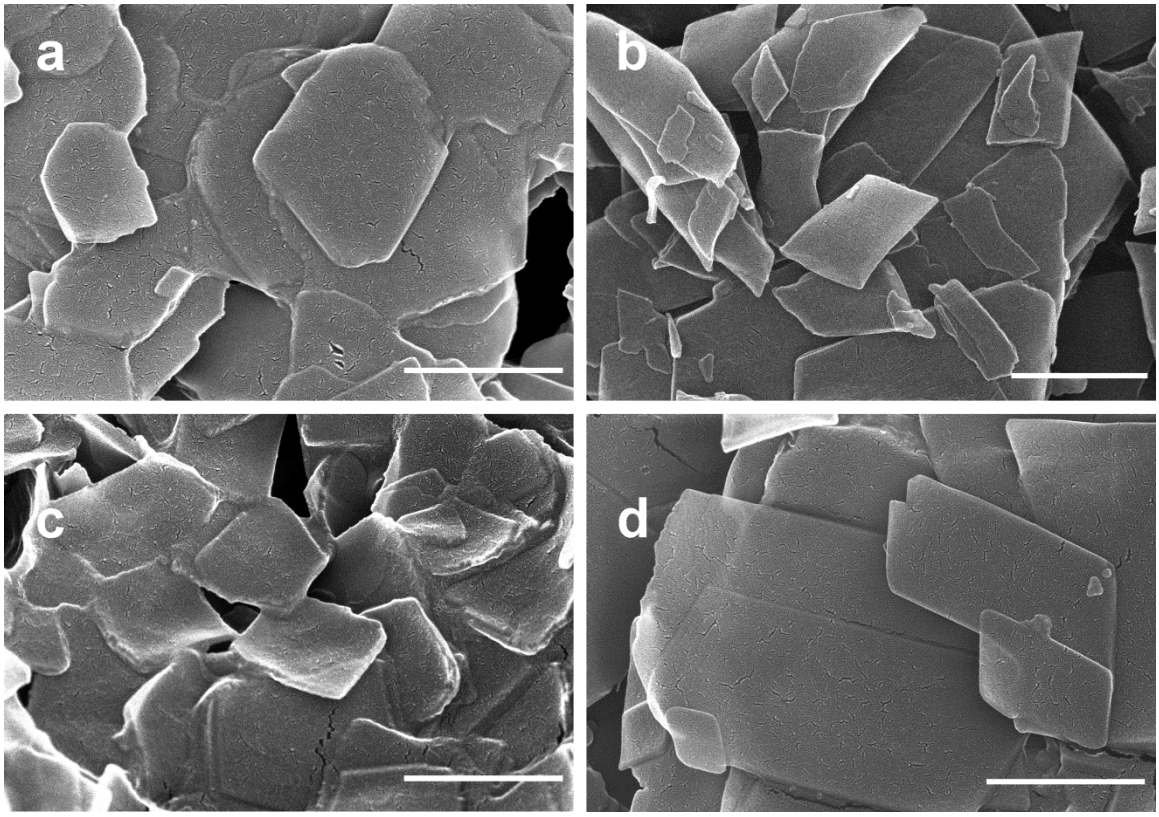

Figure 4.3. SEM micrographs of micro and submicron particles obtained from (a) Only Val /Sp60, (b) Only Val /Tw80, (c) Val and 10% Lys /Sp60, and (d) Val and 10% BSA /Sp60. The bar represents 1 μm.

It is known from previous investigations that stabilizer can arrest particle growth by impeding the coagulation or condensation processes [24,29–32]. Since surfactants are

often used as stabilizers, we explored the use of polysorbate and sorbitan derivatives, which are soluble in aqueous and organic solvents respectively [33,34]. Since the antisolvent co-precipitation is a bottom up process is critical that all components are fully dissolved prior to beginning. The direct effect of the surfactant is to increase τ_{cond} and τ_{coag} which results in a decreased of the τ_{precip} yielding smaller particle sizes [27]. As depicted in Figure 4 the presence of surfactants, allowed submicron particles to form as either blank particles (only Val) or Lys co-precipitated particles. The inclusion of BSA in this system was not successful due to the appearance of large agglomerates that were seen with laser diffraction. Lysozyme was found to be effective in hindering further particle growth upon precipitation, due to its smaller molecular weight when compared to BSA. Optimally, the use of 10% Lys in the aqueous solution further decreased Z-average from 888 nm (Val only /Sp60) to 473 nm. This is in agreement with previous findings where enzyme-coated microcrystals had smaller particle size than a crystal control [23]. The smaller particle size of enzyme-coated crystals was attributed to the precipitation of the protein on the surface of the submicron particles. We believe that this surface coverage works to slow the condensation and coagulation steps of the precipitation process limiting particle growth, much like the effect of surfactants. Additionally, polydispersity was significantly narrowed ($p<0.01$) by the use of Lys to 0.19.

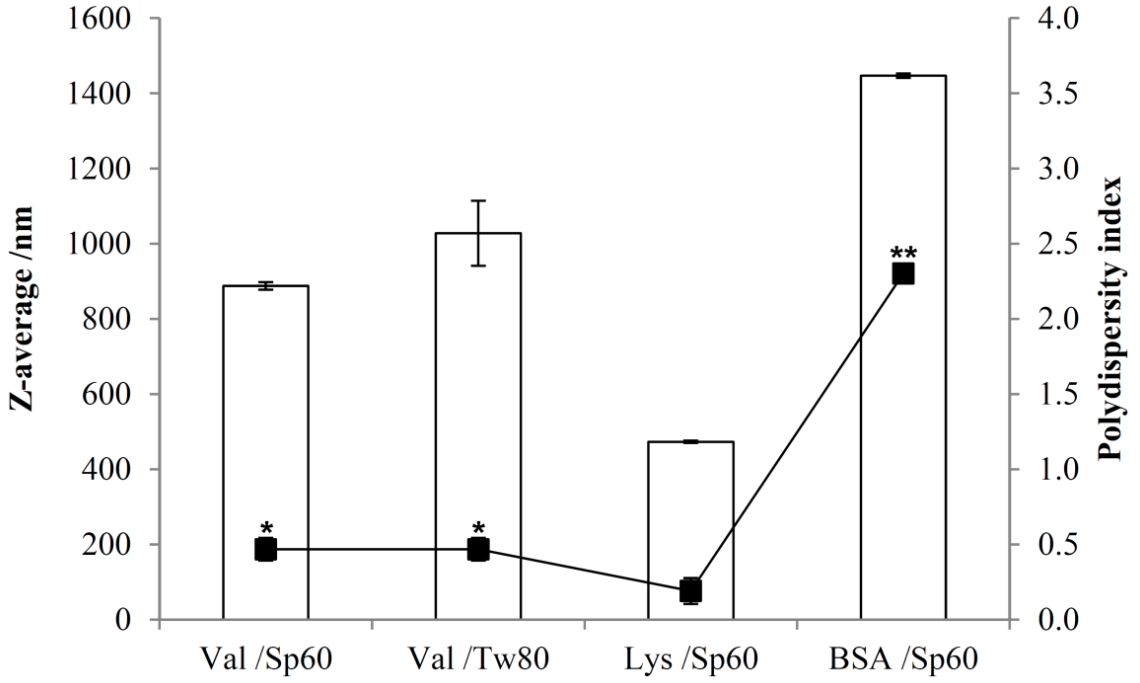

Figure 4.4. The influence of surfactant and protein model over Z-average (□) and polydispersity index (■). *: no statistical significance in difference ($p>0.5$). All other values were significantly different to a $p\leq0.01$ level. **: since BSA was determined by LD, Size is expressed as D50 and polydispersity is expressed as span here and not PdI.

4.4.3. The effect of processing variables

To investigate the importance of processing variables in the manufacture of submicron particles an aqueous formulation composed of Val 15.3 mg/mL (a quarter of 90% saturation solubility) and 10% w/w of Lys solids were used. A three factor two level full factorial DoE was designed to study the effect of sonication time, sonication intensity, and sonicating lapse duration. Sonication time was set either at 5 or 20 minutes, intensity was set either at 320 or 400 Watts, and the duration of sonicating lapse was set to either 0.3 or 0.6 seconds (Table 4.2).

Table 4.2. DoE to study the effect of process variables such as sonication time, intensity, and duration of lapse over particle size (Z-average and PdI). Results are represented as the mean and standard deviation in parenthesis.

Experiment	Sonication time /min	Sonication intensity /Watts	Sonication lapse duration /sec	Z-average /nm	PdI
SPV1	5	8	30	780.2 (50.9)	0.33 (0.03)
SPV2	20	8	30	473.2 (3.1)	0.19 (0.09)
SPV3	5	10	30	1011.4 (79.1)	0.44 (0.10)
SPV4	20	10	30	742.1 (9.4)	0.34 (0.03)
SPV5	5	8	60	1231.7 (70.7)	0.42 (0.11)
SPV6	20	8	60	961.5 (54.2)	0.57 (0.13)
SPV7	5	10	60	1194.0 (38.3)	0.37 (0.06)
SPV8	20	10	60	723.7 (60.3)	0.42 (0.09)
Effect on particle size	-329.2	56.1	276.0	--	--

After statistical analysis of the DoE all three factors were associated with significant control over the process ($p<0.02$). Sonication time was the strongest factor in terms of effect over Z-average (-329.2) and was the only factor that contributed to reducing the particle size when increased from 5 to 20 minutes (Table 4.2). Sonicating during the precipitation process reduces the mixing time, thus increasing the rate of nucleation. However, sonication also increases the overall precipitation time by impeding coagulation and condensation. Increasing both intensity and lapse duration during sonication was associated with larger particle sizes, probably due to a plateau in performance of sonication, warranting a more detailed exploration of these variables

beyond the scope of this study. From these results we can conclude that longer times, a low intensity and short duration of lapses demonstrate a high level of control for particle size in these studies. Only a decrease in the duration of the sonication lapse resulted in a significant decrease in PdI ($p<0.01$). With the exception of SPV6 all polydispersity results had acceptable values, with PdI ranging from 0.2 – 0.4 [35,36] indicating that the process overall provides narrowly distributed submicron particles (Table 4.2). It is important to note that particle shape also plays a role in the determination of particle size and size homogeneity by DLS [37]. Since, the submicron particles take on the flake-like shape of Val crystals they are asymmetric and thus they decrease homogeneity upon determination of PdI. Similar findings have been described in platelet-like solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC). Asymmetric particle shape has been found to increase PdI values ranging from 0.1 – 0.3 [37].

4.4.4. The effect of formulation variables

These findings led us to investigate the formulation variables controlling particle size. Firstly, the type of surfactant, concentration of surfactant, Val concentration, and the volume of aqueous phase added to the process were controlled in a 4 factors 2 levels fractional factorial DoE (Table 4.3).

Table 4.3. DoE to study the effect of surfactant concentration and type, Val concentration, and aqueous volume added over particle size (Z-average and PdI). Results are represented as the mean and standard deviation in parenthesis.

Experiment	Surfactant type	Surfactant conc.	Val conc. /mg/mL	Aqueous volume added /mL	Z-average /nm	PdI
SFV01_01	Span60	CMC	15.3	1	576.6 (19.2)	0.33 (0.05)
SFV01_02	Span80	CMC	15.3	1	534.2 (38.1)	0.24 (0.02)
SFV01_03	Span60	4CMC	15.3	1	929.4 (99.4)	0.66 (0.05)
SFV01_04	Span80	4CMC	15.3	1	636.7 (8.6)	0.81 (0.04)
SFV01_05	Span60	CMC	30.6	1	720.0 (21.9)	0.41 (0.04)
SFV01_06	Span80	CMC	30.6	1	696.5 (22.3)	0.39 (0.06)
SFV01_07	Span60	4CMC	30.6	1	736.3 (71.4)	0.43 (0.04)
SFV01_08	Span80	4CMC	30.6	1	735.2 (3.2)	0.37 (0.02)
SFV01_09	Span60	CMC	15.3	5	721.4 (45.5)	0.31 (0.04)
SFV01_10	Span80	CMC	15.3	5	1057.8 (142.6)	0.58 (0.03)
SFV01_11	Span60	4CMC	15.3	5	1255.0 (172.7)	0.63 (0.01)
SFV01_12	Span80	4CMC	15.3	5	977.7 (206.5)	0.17 (0.04)
SFV01_13	Span60	CMC	30.6	5	901.4 (16.9)	0.40 (0.04)
SFV01_14	Span80	CMC	30.6	5	622.5 (37.7)	0.16 (0.02)
SFV01_15	Span60	4CMC	30.6	5	630.2 (59.1)	0.21 (0.07)
SFV01_16	Span80	4CMC	30.6	5	784.3 (15.8)	0.30 (0.03)
Effect on size	-53.18	106.80	-107.78	173.18	--	--

All variables had a significant effect on particle size (Z-average, $p<0.05$). Sp60 and Sp80 were found to be similarly effective in arresting particle growth as organic soluble surfactants [38], which follows since they are from the same family of surfactants

with very similar physicochemical characteristics. Thus the surfactant types used in this study had the least effect on decreasing particle size (-53.18). In this set of experiments we also observed an increase in particle size when surfactant concentrations were increased beyond the CMC. The formation of micelles acts by decreasing the amount of surfactant molecules available to arrest particle growth in solution [27]. Decreasing the volume of the aqueous phase added, and increasing Val concentration were both associated with a decrease in D50 ($p<0.05$). Both of these factors contribute to an increase in the rate of nucleation and overall precipitation [39], consequently resulting in a reduced the final particle size, as discussed above. Polydispersity was significantly controlled by all the factors studied ($p<0.05$). When using Sp80, decreasing the concentration of surfactant, increasing the amount of aqueous volume added, as well as increasing Val concentration from 15.3 to 30.6 mg/mL were associated with a decrease in polydispersity; however, only Val concentration and the volume of aqueous volume affected the polydispersity profoundly.

By using the most relevant factors in the process and formulation variables we sought to investigate the influence of variables over the yield and activity of Lys. As stated above, proteins and peptides are labile molecules that could be rendered inactive by interacting with organic solvents, thus we measured remaining Lys activity as an indicator of structure stability following manufacture. For this, a 4 level 2 factor fractional factorial DoE was designed using Sp60 at its CMC. Val concentration was varied from 30.6 to 61.2 mg/mL, Lys concentration (Lys/%), amount of aqueous added, and total organic volume for precipitation were studied in this DoE as depicted in Table 4.4.

Table 4.4. DoE to study the effect of Val concentration, Lys loading, aqueous volume added, and organic volume over particle size (Z-average and PdI), Lys yield, and activity after manufacture. Results are represented as the mean and standard deviation in parenthesis.

Experiment	Val conc /mg/mL	Lys /%	Aq added /mL	Org vol /mL	Z-average /nm		PdI		Lys yield /%		Activity /%	
SFV02_01	30.6	10	1	100	813.7	(18.7)	0.31	(0.04)	11.2	(0.6)	97.9	(0.2)
SFV02_02	61.2	10	1	60	733.4	(2.5)	0.26	(0.06)	9.8	(0.5)	99.0	(0.3)
SFV02_03	30.6	40	1	60	757.9	(15.5)	0.38	(0.04)	21.2	(1.1)	96.4	(0.6)
SFV02_04	61.2	40	1	100	439.0	(5.2)	0.53	(0.04)	48.8	(2.4)	98.7	(0.1)
SFV02_05	30.6	10	5	60	965.9	(29.5)	0.35	(0.03)	14.6	(0.7)	94.5	(0.2)
SFV02_06	61.2	10	5	100	928.0	(51.8)	0.42	(0.02)	11.7	(0.6)	96.8	(0.5)
SFV02_07	30.6	40	5	100	699.7	(72.7)	0.92	(0.07)	105.5	(2.3)	93.7	(0.3)
SFV02_08	61.2	40	5	60	1156.3	(122.6)	0.45	(0.04)	103.3	(2.5)	97.2	(0.1)
Effect on size	-96.18	n.s.	199.91	-58.62	--		--		--		--	
Effect on Lys yield	1.42	26.8	12.78	n.s.								
Effect on activity	21.46	-35.4	65.68	-18.95								

n.s.: not a statistically significant factor.

Except for the organic solvent volume, all variables were found to significantly control Z-average ($p<0.01$). Again, decreasing the volume of the aqueous phase added had the strongest effect (199.91) in decreasing particle size. A high volume of aqueous solution not only provided more precipitant for the process, increasing the rate of nucleation and decreasing the precipitation time by favoring coagulation and condensation resulting in larger particles. As observed above, increasing Val

concentration resulted in a decrease in particle size in terms of Z-average. Increasing the concentration of Lys in the aqueous phase resulted in a decrease in particle size. As previously described, as the enzyme precipitates on the surface of the growing crystals it stabilizes and hinders further particle growth. However, increasing the concentration of lysozyme was associated with an increase in PdI. This could be correlated with potential self-aggregates of lysozyme at higher concentrations. Normally, increasing the concentration of lysozyme does not render aggregates on the larger side of the particle sizes (few microns) but mostly in the few hundreds of namometers range. On the other hand, increasing the aqueous volume added to the precipitation process resulted in the presence of large particles. Increasing the volume of organic phase used only slightly correlated with an increase in PdI.

Increasing the concentration of Lys in the aqueous phase had a strong positive impact in the final yield of lysozyme in the precipitated particles. Similarly, a strong positive effect can be attributed to aqueous volume added to the precipitating system. A slight (yet significant) positive effect can be seen for the amino acid concentration, although this does not have a strong control over the final lysozyme content. All factors studied had significant effects in controlling the remaining activity of Lys-containing particles, and remaining activity after manufacture was excellent for all formulations studied. All final activity results were above 93%, and except for two, they ranged from 96 to 99% remaining activity (Table 4.4), revealing that the remaining Lys after the antisolvent co-precipitation manufacturing process is almost completely active compared to a control stock solution of Lys. This is in agreement with previous reports on enzyme or DNA-coated microcrystals obtained in a similar fashion that resulted in high activity

and stability of the biologics after manufacture [17,22,23,28]. This shows that although the use of a probe sonicator imparts high energy mixing to the process of precipitation, the immobilization of protein to the amino acids crystals imparts enhanced stability to the molecule. Moving forward from this investigation we expect to optimize the process around SFV02_04 which while maintaining small particle size (439 nm) provided adequate yield (around 50%) and high activity after manufacture (98.7%).

4.5. CONCLUSION

This novel method of manufacture can yield stable protein-loaded submicron particles with suitable control over particle size and a narrow size distribution range. Micro and submicron particles obtained in this investigation conformed to the original shape of pure valine crystals, demonstrating that the addition of the protein/peptide to the system did not affect the morphology of particles following their subsequent precipitation on to its surface.

The combined use of a nebulizer and probe sonicator drastically decreased particle size and resulted in narrow particle size distributions. In addition, the use of 2-propanol was the most effective antisolvent investigated. Additionally, the use of a surfactant to stabilize particle growth allowed us to adequately control the manufacture of protein/peptide submicron particles. By studying various factors using a design of experiments strategy (DoE) we were able to establish the critical controlling factors for this new and improved method of manufacture. This optimized method of manufacture was sought to be further adapted to more therapeutically relevant actives, such as insulin, and its use is decribed below in Chapter 6.

4.6. REFERENCES

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5. Protein-coated Nanoparticles Embedded in Films for Buccal Delivery[4]

ABSTRACT

The objective of this chapter was to develop a mucoadhesive film for the delivery of lysozyme as a model protein and to render the enzyme stable and homogeneously distributed in the film by using a newly developed process for the manufacture of Lys-loaded submicron particles. For this, an antisolvent co-precipitation process was used to manufacture Lys-loaded submicron particles. After size, yield, and stability characterization the selected batch of particles was further formulated into polymeric films. Polymethacrylates and HPMC were used as matrix formers and films were obtained by a solvent casting process. Films were characterized for mechanical properties, mucoadhesion, Lys release kinetics and activity after manufacture. It was found that protein-coated nanoparticles particles (PCNP) could be obtained at pH 6.8. Formulation SPH02 had a z-average of 347.2 nm, a zeta potential of 21.9 mV, and 99.2% remaining activity after manufacture and was selected for further study. Films with Eudragit RLPO (ERL) exhibited excellent mechanical and mucoadhesive properties and the release of Lys could be tailored by the addition of HPMC in the polymeric matrix. FPH04 consisted of equal amounts of ERL and HPMC and revealed a sustained release over the 4 hours period of time of the study with Lys remaining fully active. Thus, successful development of film formulations containing Lys-loaded particles was achieved. All Eudragit RL films had acceptable mechanical properties, excellent

[4] Significant portions of this chapter were taken from: J.O. Morales, A.C. Ross, J.T. McConville, Protein-coated nanoparticles embedded in films for buccal delivery, Journal of Pharmacy and Pharmacology. (2012) *Submitted*.

mucoadhesive properties, and excellent remaining Lys activity. Moreover, the use of HPMC allowed for tailoring the profiles in sustained release formulations.

5.1. INTRODUCTION

As earlier mentioned, the recent increase in the number of products under review by the FDA or undergoing late phase clinical trials demonstrates the fact that protein and peptide therapeutics is a rapidly growing field in the pharmaceutical industry [1]. However, it is widely reported in the literature that the efficacious delivery of these therapeutic agents can become the determinant factor in product development [2,3]. Therefore, in order to achieve the real potential of protein and peptide therapeutics, effective smart delivery platforms and strategies, to overcome the formulation and delivery challenges, need to be developed. The conventional approach for the delivery of macromolecules is through injections [4,5]. This method of delivery is largely associated with drawbacks in patient compliance and acceptance. The start of therapies can get delayed and patients can develop needle anxiety [6]. In addition, the number of injections may lead to compliance issues [7] in therapies that rely on this route of administration. Therefore, alternative routes of delivery are vital to achieve successful product development.

Among alternative routes of delivery for proteins and peptides, the oral route has been for a long time widely investigated [8–10]. However, several drawbacks in the gastrointestinal tract (GIT) make the development of novel delivery platforms for macromolecules very challenging. Instability in gastric pH, proteolytic enzyme content in the upper GI tract, and insufficient permeation and bioavailability has limited the success that has been achieved [11]. These limitations have led to the exploration of other routes of delivery [12,13], such as pulmonary, nasal, and transdermal routes. Particularly, the buccal route of delivery offers interesting advantages in comparison to the oral route and

the GIT limitations for the delivery of protein and peptides [11]. In bypassing the absorption via the gut, the stability of macromolecules is not compromised before reaching circulation. Other advantages of this route of delivery such as its good vascular drainage, ease of administration, and relatively low enzyme levels make it a good candidate for the delivery of proteins and peptides [14].

Mucoadhesive films as dosage forms for the buccal route of delivery have been investigated in the past decade but little efforts have been done with regards to the delivery of proteins and peptides in particulate forms [15,16]. From a formulation standpoint, actives are usually added to the film by their inclusion to the casting solution, then allowing it to dry into the solid form. However, in general the polymers utilized in formulations containing proteins are more hydrophobic in comparison to the hydrophilic nature of proteins [10]. This could potentially lead to separation and/or aggregation during storage, or in vivo, leading to possible instability [17]. Additionally, strategies like incorporating insulin as a solid solution into PLGA microspheres, to prevent chemical reactions in the solid state and to control the peptide release, have been unsuccessful [18]. During PLGA erosion, the microenvironmetal pH drops and deamidation has been found to be the main reaction that causes insulin instability [19]. For the delivery of insulin, chitosan seems to be a more suitable candidate as a polymer vehicle. Cui et al. (2009) have developed chitosan-ethylenediaminetetracetic acid (chitosan-EDTA) films containing insulin for buccal delivery and have demonstrated the retention of the physical structure of the peptide upon release [20]. However, there is no mention of the uniformity of the drug in the film upon solidification which prohibits any conclusion about drug distribution homogeneity. More recently, Giovino et al. (2012) have developed chitosan

films for the buccal sustained delivery of insulin in PEG-b-PLA nanoparticles as a model for buccal macromolecular delivery [21]. Although adequate physico-mechanical properties were achieved, very high heterogeneity was revealed by the mechanical variables studied (time to break, tensile strength, Young's modulus, and work done to break). This therefore raises concern over the tight control of manufacture necessary to prepare films with homogeneous particle distribution, adequate physico-mechanical properties, high yield and retention of macromolecule activity.

In recent years, investigations of enzyme immobilization in organic solvents have opened the door for the manufacture of particulates-containing films with enhanced activity. In particular, the antisolvent co-precipitation method has been shown to produce particles coated with a variety of biologicals including nucleic acids [22], proteins [23], enzymes [24], and other particulate systems [25]. However, most of these investigations lead to particles in the range of 1–5 μm or higher. To guarantee physical stability of the films in terms of mechanical and also mucoadhesive properties, such large particles are undesired due to potential for aggregation and loss in active distribution homogeneity [26]. Our group has recently published on the manufacture of submicron and nanosized particles of lysozyme (Lys) loaded D,L-valine (Val), also known as protein-coated nanoparticles (PCNP), and the advantages of this method of manufacture to provide high yield and enzymatic stability [27]. Based on previous investigations, it is known that a combination of high mixing energy provided by a probe sonicator, the addition of the aqueous phase by means of a nebulizer, and the use of surfactant as a stabilizer, can altogether yield relatively narrowly distributed submicron protein-containing particles. Here, after studying the effect of aqueous phase pH on particle size, we selected an

optimized formulation for the manufacture of films. Mucoadhesive films intended for the buccal delivery of macromolecules were developed using Lys as model to yield high enzyme activity after manufacture and controlled release in vitro.

5.2. MATERIALS

D,L-valine (Val) and lysozyme (Lys) were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, Missouri). Sorbitan monostearate (Span60) was obtained from Spectrum (Spectrum Chemical, New Brunswick, New Jersey). Eudragit RSPO and RLPO (ERS and ERL) were kindly donated by Evonik (Evonik Industries, Darmstadt, Germany). Carbopol® 974P (C974P) and Noveon® AA-1 Polycarbophil (PCP) were donated by Lubrizol (Lubrizol Advanced Materials, Cleveland, Ohio). Hydroxypropyl methylcellulose (HPMC, Methocel E50 Premium LV) was donated from Colorcon (Colorcon, Harleysville, Pennsylvania). Triethyl citrate (TEC; Vertellus Specialties Inc, Indianapolis, Indiana), mucin (Spectrum Chemical, New Brunswick, New Jersey), and Micrococcus lysodeikticus (Worthington Biochemical Corp., Lakewood, New Jersey) were purchased and used as received. HPLC-grade 2-propanol (IPA) was obtained from Fisher Scientific (Fisher Scientific, Fair Lawn, New Jersey) while deionized water was procured in house. All other chemicals used were of analytical or reagent grade.

5.3. METHODS

5.3.1. Protein-coated nanoparticle (PCNP) manufacture

The manufacturing process was based on antisolvent co-precipitation and our approach has been recently published elsewhere [27]. Briefly, the co-precipitant Val and the amount of Lys to be precipitated were dissolved in one the buffers and solutions

studied (Table 5.1). First, Val was dissolved in the aqueous phase at a concentration of 61.2 mg/mL (or 90% of its saturation concentration) and then Lys was dissolved in this solution to yield a protein content of 40% w/w based on solid content. By means of an Aeroneb Pro® vibrating mesh nebulizer (Aerogen, Galway, Ireland), the aqueous phase is then added to the antisolvent organic phase. The organic must be miscible with water in order to promote the fast dehydration of the precipitant and co-precipitant. We have shown previously that IPA containing Span60 is the most effective antisolvent yielding smaller particle sizes[27]; therefore, a 0.008 mM Span60 solution was utilized. Finally, during the addition of the aqueous phase high energy mixing is provided by means of a Branson Sonifier 450 probe sonicator (Branson Ultrasonics, Danbury, Connecticut). After addition of the total volume of aqueous phase sonication is maintained for 20 more minutes to further stop particle growth during the early stages of coagulation [27].

Table 5.1. Formulations prepared to study the effect of pH in the manufacturing process of Lys PCNP.

Formulation	Protein model	Buffer /solution	pH
SPH01	Lys	N Phthalate	5.4
SPH02	Lys	Phosphate	6.8
SPH03	Lys	Borate	10
SPH04	Lys	NaOH	13

5.3.2. Particle sizing

To determine the particle size of the slurries obtained in IPA a Zetasizer Nano ZS (Malvern Intruments Ltd., Worcestershire, UK) was utilized. Mean particle size was obtained as a z-average which corresponds to the intensity weighted mean hydrodynamic

size measured by dynamic light scattering (DLS). Additionally, an estimate of the width of the distribution was obtained from the instrument as a polydispersity index. Approximately 1 mL of IPA slurry was analyzed by DLS in 1cm path length disposable polystyrene cuvettes. A total of 3-5 determinations of 15-20 runs each were conducted.

5.3.3. Zeta potential

Zeta potentials (ZP) of slurries were obtained by laser Doppler micro-electrophoresis using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Approximately 1 mL of slurry was added to a polycarbonate capillary cell for determination of zeta potential. A total of 5 determinations of 14–20 runs each were conducted to obtain the average zeta potential of the slurries.

5.3.4. Lysozyme quantification by RP-HPLC

Chromatography was performed using a Zorbax 300SB® C18 Rapid Resolution column (3.5 μm, 4.6 mm ID x 150 mm length) (Agilent Technologies, Santa Clara, CA).The mobile phase consisted of two solvents with different polarities: solvent A consisted of water with 5% v/v acetonitrile and 0.1% v/v trifluoroacetic acid, while solvent B consisted of acetonitrile with 5% v/v water, and 0.085% v/v trifluoroacetic acid. The mobile phase consisted initially of 10% v/v solvent B with a solvent gradient of 60% v/v solvent B in 16 minutes. The flow rate was set to 1 mL/min and temperature remained constant at 25 °C. The injection volume was 50 μL and the UV detector was set to 215 nm. For the determination of Lys content on the slurries, particles were separated by centrifugation at 12,000 rpm then dried overnight at room temperature with an air current. The solids were then resuspended in pH 6.8 buffer and quantified with the RP-

HPLC method. To determine the content of Lys in films, release of Lys was allowed to occur over 24 hours at 37 °C in an orbital shaker and then the media assayed by RP-HPLC.

5.3.5. Lysozyme activity with Micrococcus lysodeikticus

The enzymatic activity of lysozyme after manufacture of particles was determined turbidimetrically based on the Shugar method [28]. Activity is correlated with a decrease in absorbance at 450 nm of solutions containing Micrococcus lysodeikticus due to the lytic activity of lysozyme on the cell walls. A 0.3 mg/mL cell suspension (0.9 mL) was mixed with a stock lysozyme pH 6.2 phosphate buffer solution containing 0.1 mg/mL (0.1 mL) to determine the maximum lytic effect. After separation and drying of particles, the solid was dissolved in a pH 6.2 phosphate buffer to a concentration of 0.1 mg/mL. Following the same procedure, sample solutions were assayed against a M. lysodeikticus suspension and absorbance was measured at 450 nm to determine maximum activity of solutions. Relative activity was calculated considering the absorbance measured for a fresh Lys stock solution as 100% activity. To determine the remaining relative activity of Lys in films, the same media obtained after overnight shaking depicted in section 5.3.4 was utilized.

5.3.6. Preparation of particle-containing films

Casting solutions were prepared by combining two organic solutions and cast overnight in PTFE molds. Acetone was used to dissolve or suspend the polymer combinations as depicted in Table 5.2. This solution was combined in a 4:6 acetone to IPA ratio with suitable amounts of SPH02 (pH 6.8) of Lys-containing IPA (for the

control formulation, FPH06) to yield the final casting solution. After 24 hours, films were peeled off and stored in aluminum foil sachets in a dessicator until characterization.

Table 5.2. Film formulation compositions (as % w/w) that were studied to investigate drug release and uniformity of films containing SPH02.

Formulation	Eudragit RL	Eudragit RS	HPMC	TEC	IPA solution
FPH01	90	--	--	10	SPH02
FPH02	73	--	17	10	SPH02
FPH03	64	--	26	10	SPH02
FPH04	45	--	45	10	SPH02
FPH05	--	90	--	10	SPH02
FPH06	90	--	--	10	Lys*

*: Unprocessed Lys was dispersed in IPA to prepare the control formulation FPH06.

5.3.7. Morphology of particles and films by SEM

A scanning electron microscope Quanta 650 FEG (SEM, FEI Company, Hillsboro, Oregon) was used for imaging and ultrastructure analysis of both particles and particle-containing films. After separation and drying of slurries, samples were mounted onto aluminum stubs using conductive carbon tape for coating. For the imagining of films, cross-sections were obtained by a freeze fracture method to ensure clean-cut edges and to avoid plastic deformation (often resulting from mechanical cutting). Fragments of the surface of the film were frozen by submerging in liquid nitrogen and thus cracked by freezing. Pieces of the films were fixed on aluminum stubs by means of conductive carbon tape for coating. Coating was performed using a 208 HR Cressington sputter coater (Cressington Scientific Instruments Ltd, Watford, UK) with Pt/Pd to a thickness of

10–15 nm in a high vacuum evaporator. To avoid structural deformation during imaging, the electron beam voltage was kept at 2–5 kV [29].

5.3.8. Mucoadhesive and mechanical properties of films in vitro

Mucoadhesion tests were conducted on a TA.XTPlus texture analyzer (Stable Micro Systems, Godalming, UK) equipped with a 5 Kg load cell. Briefly, films were held in the horizontal position and 5 μL of model mucus (a freshly made 2% w/v mucin solution) was placed on top of the film. This amount is sufficient to mimic the average saliva thickness [30]. A 7 mm diameter stainless steel cylindrical probe was attached to the mobile arm of the texture analyzer and it was brought in contact with the film and mucin solution, held at an applied force of 50 mN for 15 seconds and then withdrawn at a 0.5 mm/second rate. Mucoadhesive force (MAF) and work of adhesion (WoA) were obtained from the peak and the area under the curve in the force versus distance profile, respectively.

For the determination of mechanical properties, rectangular strips of 1 x 5 cm^2 were cut and 1 cm on each end was held between clamps attached to the texture analyzer, leaving a testing area of 1 x 3 cm^2. The upper clamp (connected to the mobile arm of the texture analyzer) was moved upwards at a rate of 0.5 mm/sec until film failure. Stress is obtained from the force measurements obtained from the instrument divided by the cross-sectional area of the film, while strain is computed by dividing the increase in length by the initial film length. From the plot, the tensile strength (TS) and the elongation at break (EB) are obtained from the peak stress and the maximum strain, respectively, also represented by the following equations [16]:

$$Tensile\ strength\ (TS) = \frac{Peak\ stress}{Cross-sectional\ area\ of\ film}$$

$$Elongation\ at\ break\ (EB) = \frac{Increase\ in\ length\ at\ break}{Initial\ film\ length} \times 100$$

Additionally, the elastic modulus (EM) was obtained from the initial elastic deformation region in the stress vs. strain plot [31]. Since the rate of the mobile arm extension was constant for all samples tested, direct comparison of the slope in this region can be done. To further evaluate mechanical properties three additional parameters were computed from the conventional mechanical parameters obtained from the plot as follows [32]:

$$Tensile\ strength\ to\ modulus\ ratio = \frac{TS}{EM}$$

$$Relative\ surface\ energy\ (RSE) = \frac{TS^2}{2 \times EM}$$

$$Toughness\ index\ (TI) = \frac{2}{3} \times TS \times EB$$

5.3.9. Lysozyme release and kinetics analysis

Lysozyme release was performed on Franz diffusion cells with phosphate buffer pH 6.8 as media. To support the films and avoid solid disintegration into the receiving chamber a 0.1μm nylon membrane filter was additionally placed between the donor and receptor compartment. We found that said pore size does not limit diffusion; therefore, it

does not have an impact in the release properties from the films (data not shown). Films were cut into 1.5 cm diameter circular samples (n=3) and allowed to release into the reservoir media for 4 hours. At intervals of 0.25, 0.5, 1, 2, 3, and 4 hours 300 μL samples were withdrawn and replaced with fresh media. Lysozyme content was determined with the method described in section 5.3.4.

To analyze the mechanisms involved in the lysozyme release, kinetics models were compared to the release profiles. The Higuchi [33], Korsmeyer-Peppas [34], and first order kinetic models were used to fit the data and were compared on the basis of R^2 adjusted [35]. The evaluation of the drug transport mechanism was addressed in accordance with the Korsmeye-Peppas model.

5.3.10. Statistical analysis

All statistical analyses were performed with the software Minitab Release 14® (Minitab Inc., State college, PA). One-way ANOVAs were used for multiple comparisons and Tukey's post hoc pairwise comparisons were performed to compare which results led to significant differences. For the evaluation of the kinetics models and calculation of adjusted R^2 values the software Origin® 8.0 (Northampton, MA) was used to perform the non-linear regressions for each kinetic model equation. All values are reported as the mean and standard deviation of the mean shown in parenthesis.

5.4. RESULTS AND DISCUSSION

5.4.1. The effect of pH on the particle manufacturing process

We have previously reported on a method of manufacture of submicron sized and nanosized particles containing Lys by an antisolvent co-precipitation method [27]. The

optimized method of manufacture consisted of the use of a nebulizer to add the aqueous phase into the surfactant containing organic phase under a high-energy mixing input generated by a probe sonicator. Here we investigated the effect of pH in the aqueous phase containing Lys over particle size, yield, and stability among others.

A narrow particle size distribution was obtained at optimized conditions. Due to a limit of solubility of Val and supersaturation in the aqueous phase upon addition of Lys we were unable to manufacture SPH01 particles. SPH02 at pH 6.8 was found to be the best condition for the precipitation of Lys. This formulation yielded very small particle sizes (347.2±16.9 nm), adequate PdI, and low variability (Table 5.3). The flake-like shape of particles obtained for SPH02 are in agreement with previous findings obtained by inspection under the SEM (Figure 5.1) [27]. The ZP found for SPH02 is well correlated with particles in the nano size and the magnitude indicates good stability of the formulation [36]. This explains observations during a one week period of time where particles in the slurry remained in suspension in comparison with any other formulation that sedimented shortly after manufacture.

Table 5.3. Particle size reported as z-average, polydispersity index and zeta potential of Lys formulations. Results are represented as the mean and standard deviation in parenthesis.

Formulation	Z-average /nm*	Polidispersity index*	Zeta Potential /mV
SPH01	--**	--**	--**
SPH02	347.2 (16.9)	0.36 (0.02)[i]	21.9 (3.7)[a]
SPH03	1384.0 (152.7)	0.28 (0.13)[ii]	18.3 (2.0)[a]
SPH04	1220.2 (426.6)	0.43 (0.13)	10.1 (1.2)

*: Among parameters, all differences were statistically significant ($p<0.05$). a, b: Among parameters, non-significant differences are indicated in pairs of letters, all other differences are significant ($p<0.05$). **: co-precipitant and Lys did not dissolve at pH 5.4.

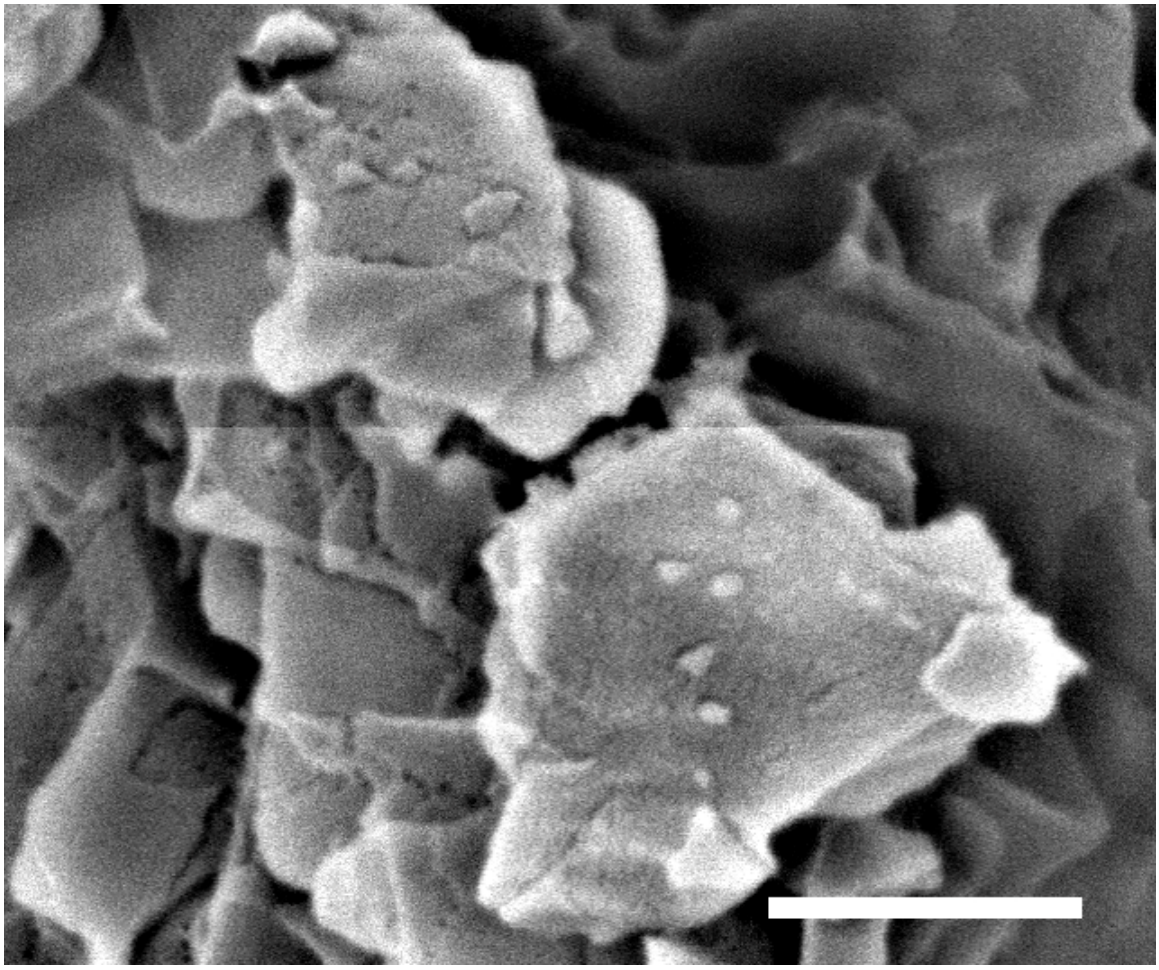

Figure 5.1. SEM micrographs of protein-loaded submicron particles from formulation SPH02. The bar represents 1 μm.

Regardless of the pH, excellent Lys yield and stability was achieved. With yields in the range of 70.5-73.4 (no statistical differences found, $p > 0.05$) and remaining relative activity in the range of 91.4-101.1 % we corroborated that the method of manufacture of nanoparticles by the antisolvent co-precipitation method is successful in rendering functional particles. This also indicates that the pH of the buffer solution containing Lys before manufacture had little effect on the resulting stability after manufacture.

Investigations on the manufacture of microparticles in the range of 1–10 μm obtained through a similar process of antisolvent co-precipitation have shown positive results regarding the stability of the macromolecules coating an inert core [37,38]. Our findings here constitute an improvement over the particles obtained previously where the optimized conditions allowed for a z-average of 439 nm with a yield around 50 % and remaining activity of 98.7 % [27]. We were also able to obtain nanoparticles with a much higher enzyme load (40 % instead of 10 % w/w) which represents an advantage in terms of dosing in the final dosage form.

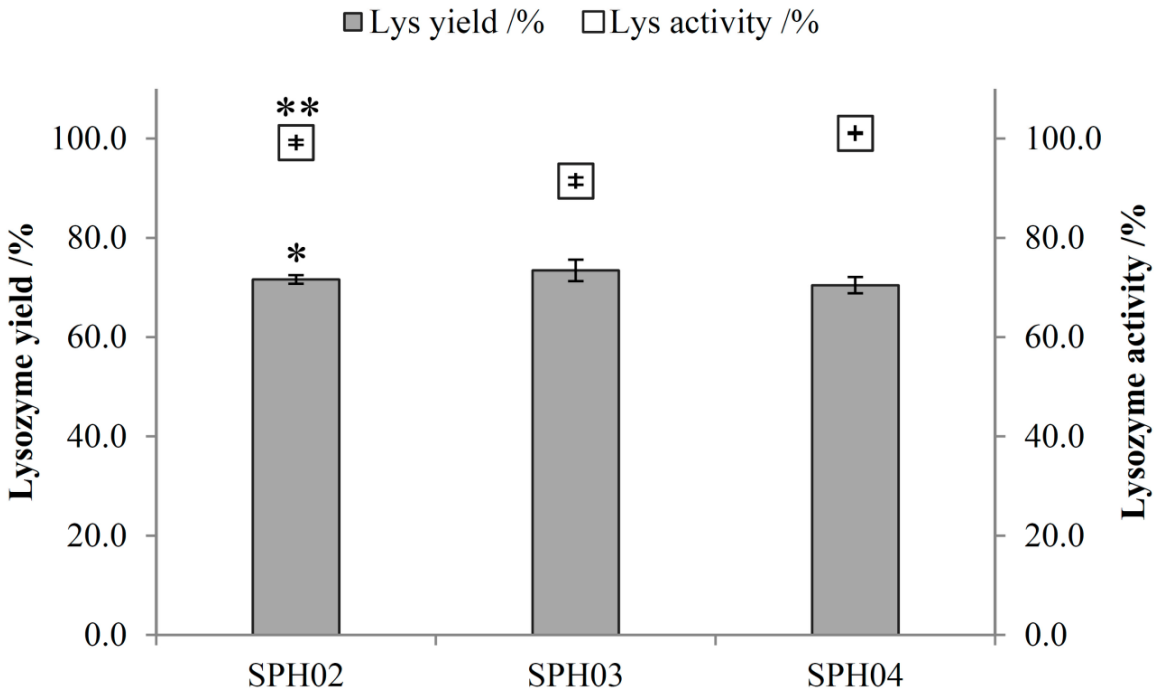

Figure 5.2. Lys yield (■) and relative activity (□) of Lys-containing particle formulations.
*: No significant differences were found among Lys yield results ($p<0.05$).
**: All the activity results were significantly different from each other to a $p<0.05$.

5.4.2. Development of Lys particle-containing films

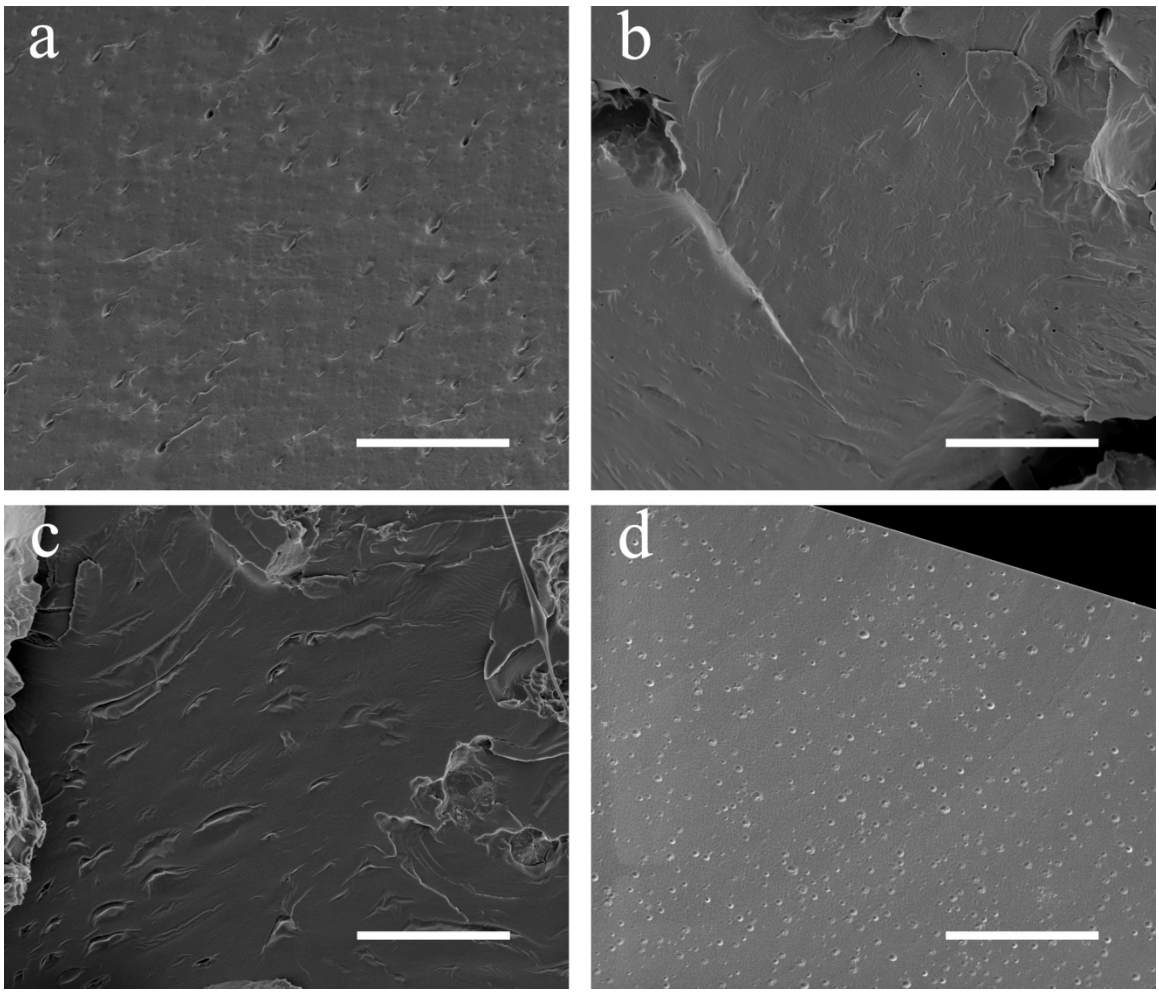

Figure 5.3. SEM micrographs of cross-sections of films obtained by freeze-fracture. (a) FPH01, (b) FPH03, (c) FPH04, and (d) FPH05. The bar represents 20 μm.

Films were successfully manufactured and their surface appeared homogeneous to the eye. An SEM observation of cross sections of selected film formulations obtained by freeze fracture reveals a uniform distribution of the flake-like particles throughout the polymeric matrix (Figure 5.3). A closer look (Figure 5.4) shows that mostly individual

particles are separately enclosed resulting in high drug content uniformity in the films. Agglomerates of HPMC can also be observed homogeneously distributed under the SEM, indicating the composite nature of the film.

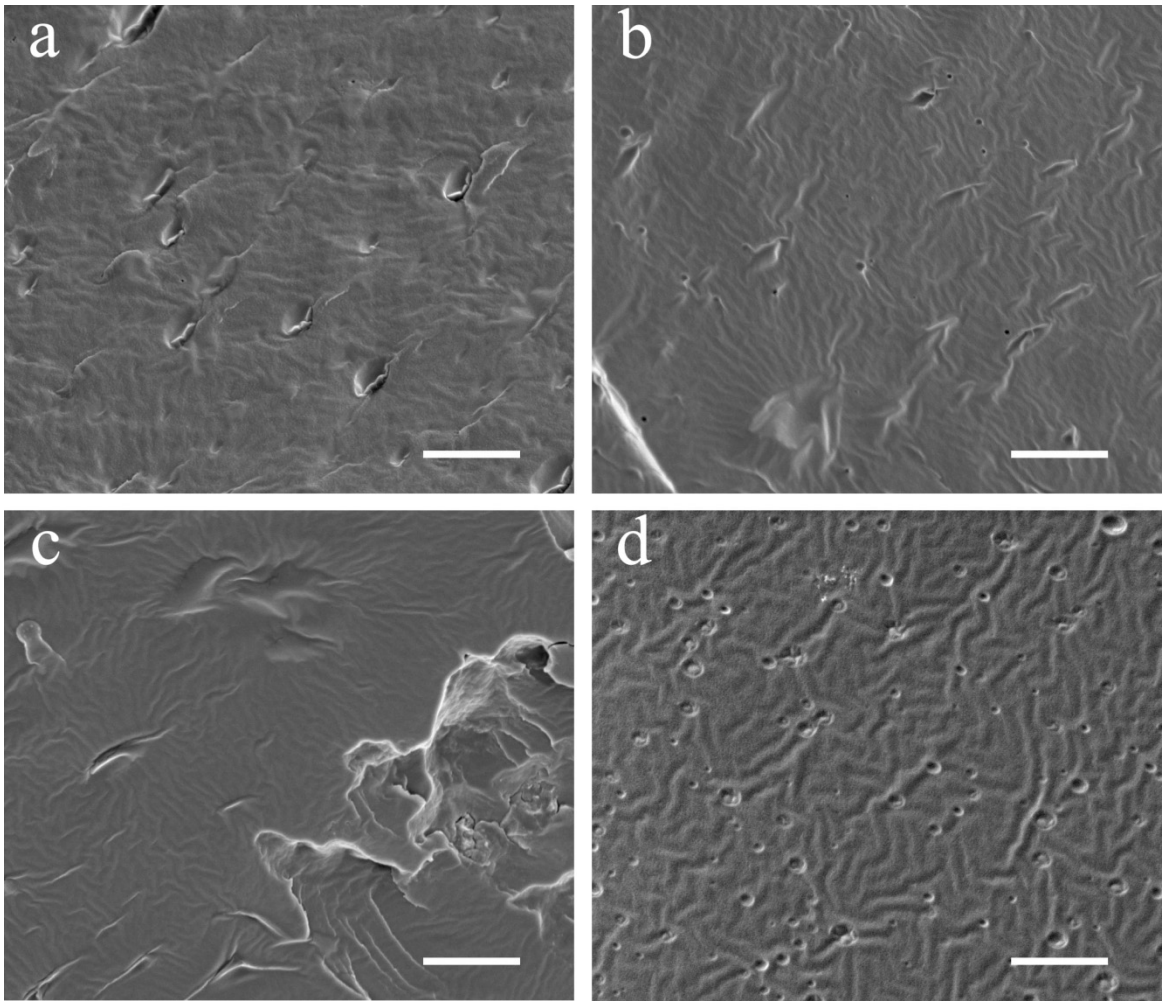

Figure 5.4. SEM micrographs of cross-sections of films obtained by freeze-fracture. (a) FPH01, (b) FPH03, (c) FPH04, and (d) FPH05. The bar represents 5 μm

5.4.2.1. Mucoadhesion and mechanical properties of Lys-containing films

ERS and ERL are more commonly known for their applications on sustained drug delivery by controlling drug release rate of dosage forms. However, more recently we have reported on the high mucoadhesive properties exhibited by polymethacrylates, and more specifically ERL [26]. In accordance with what we previously found, films containing ERL showed high or very high mucoadhesive properties. When ERL was used as the only polymeric material the MAF achieved was higher (but not statistically different, $p>0.05$) and the WoA was significantly higher than any of the other formulations studied (Figure 5.5). We believe that the presence of water-soluble particles homogeneously distributed among the film surface allows for a more rapid and homogeneous water penetration. According to the theories of mucoadhesion based on diffusion [39] and water penetration [40], the presence of water in the interface is paramount for the establishment of the mucoadhesive bond. The water rapidly driven in by the water-soluble molecules allows for polymer chain mobility resulting in entanglement with the mucin molecules in the mucus layer and establishment of the mucoadhesive bond. Similar effects have been found in systems where the drug was incorporated as particulate material. Panomsuk et al. have described that the inclusion of a water soluble drug, such as theophylline, increases the amount of water associated with the polymer favoring gelling and swelling [41]. It has also been indicated that the presence of particulate material interrupts the polymer matrix continuum, allowing the polymer chains to move more freely leading to an increase in water penetration [42]. The absence of particulate material in the formulations containing only C974P and PCP leads to their inherent mucoadhesion that results from the capacity of the polymer to absorb

water and plasticize the polymer chains to interact with mucin [39]. It should be noted that the addition of HPMC hinders the full extent of mucoadhesion enhancement possibly by capturing the particles (that are more hydrophilic) in HPMC-rich domains. This results in slower hydration and thus a weaker mucoadhesive bond that mostly depends on the mucoadhesion of HPMC. HPMC has been used in the past as a mucoadhesive material but its mucoadhesive power is lower than that observed for PCP and C974P [43,44]. A similar trend was found in investigations by Wong et al. performed on films composed of a polymethacrylates and HPMC [45]. The authors found that an increase in the HPMC concentration resulted in a decrease in mucoadhesion. We believe that higher contents than 30% make a substantial change in the material properties and the inherent mucoadhesivity of HPMC starts to play a role at high contents. Finally, ERS exhibited higher MAF than results of ERL films with drug in solid solution [26]. This in interesting considering that ERS is the more hydrophobic material due to its lower content of quaternary ammonium groups. We have previously shown that ERS consistently exhibits lower MAF and WoA in comparison to ERL [26]. However, we believe that the enhancing effect of the water-soluble particulate material discussed earlier is responsible for this higher extent in mucoadhesive properties.

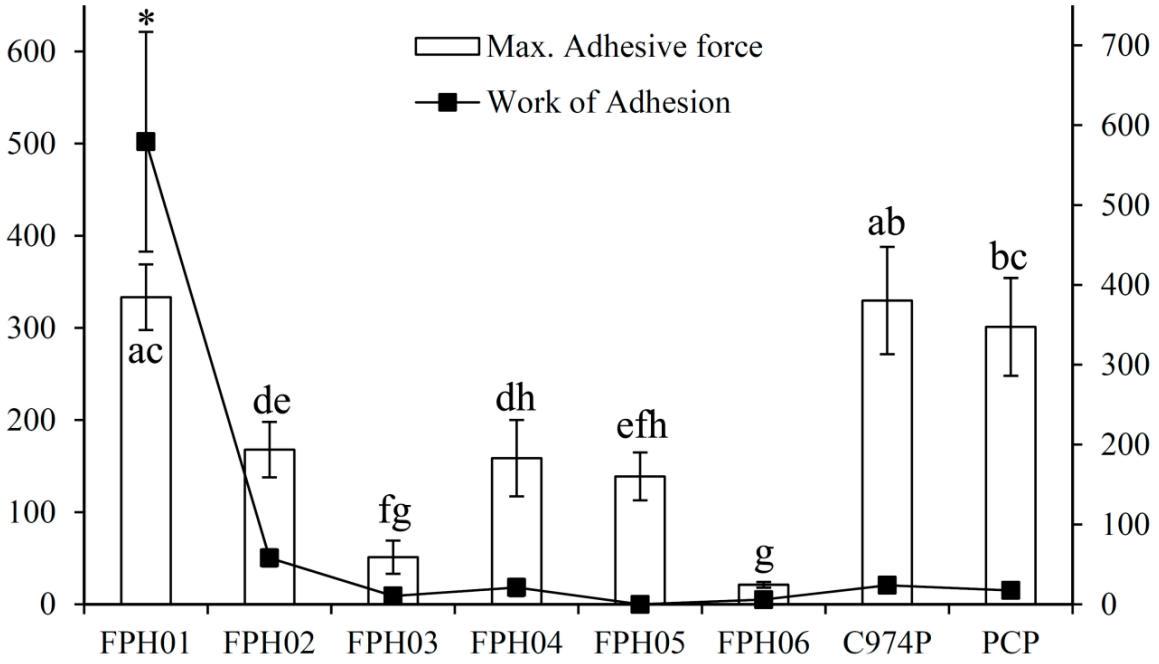

Figure 5.5. Mucoadhesive properties (□) MAF and (■) WoA for Lys-containing films. The same values for conventional mucoadhesive polymers such as C974P and PCP are depicted for comparing the performance of films developed here. a – h: Non-significant differences among MAF are indicated in pairs of letters ($p>0.05$). *: Only WoA of FPH01 was significantly different from other formulations ($p<0.05$). All other values were not statistically different ($p>0.05$).

Films as dosage forms for the buccal route of delivery have the need to withstand the stress originating from mouth mechanical activities. Both mechanical and mucoadhesive adequate characteristics are needed for the films to remain in contact with the mucosa for the desired amount of time of release [46]. Furthermore, another source of mechanical stress originates from the processes of manufacturing, handling, and administration [47]. Thus, in order to successfully develop films as dosage forms for buccal delivery it is desired a relatively high TS, EB, and a low EM [46]. Additionally,

derived from the conventional parameters extracted from stress vs. strain curves, a relatively high TS/EM, RSE, and TI are desired [32,48].

Table 5.4. Mechanical properties for Lys-containing films. Results are represented as the mean and standard deviation in parenthesis.

Formulation	Tensile strength N/mm^2		Elongation at break %		Elastic modulus $N/mm^2/\%$	
FPH01	1.653	(0.160)[a]	197.9	(26.7)[a]	0.318	(0.110)[a,b]
FPH02	2.783	(0.133)	50.0	(11.0)[b,c]	0.831	(0.048)
FPH03	5.169	(0.462)[b]	25.6	(6.5)[b,d]	1.554	(0.191)
FPH04	5.005	(0.464)[b]	18.0	(4.0)[c,d]	1.228	(0.129)
FPH05	0.580	(0.075)[c]	233.6	(43.9)[a]	0.153	(0.038)[a]
FPH06	1.273	(0.124)[a,c]	124.7	(12.9)	0.465	(0.093)[b]

a – d: Among parameters, non-significant differences are indicated in pairs of letters.

In Table 5.4 we can observe that adequate control over TS, EB, and EM was achieved for FPH01, FPH05, and FPH06, all of which only had either ERL or ERS and no other polymer. Quaternary ammonium polymethacrylates have been previously described to have suitable properties as film forming material for dosage forms for the buccal route [26]. In that study we showed that film formulations containing 10% triethylcitrate (TEC) as plasticizer rendered films with medium TS, high EB, and low EM. Here, we have found similar conditions for films that did not contain HPMC as a release modifier polymer. The addition of HPMC was correlated with an increase in TS, decrease in EB, and a slight increase in EM (Table 5.4). This is an indicative of less ductile yet more resistant films. The effect of HPMC over the mechanical properties of films is clearer after analysis of the derived mechanical parameters. TS/EM in an

indicator of the level of internal stress in a film. The larger its value the higher the film crack resistance. RSE is also utilized to estimate crack resistance and is approximated from the surface energy of the film. Finally, TI is an estimation of energy absorbed per unit volume of film under stress [32]. FPH01 is the formulation that possessed the largest TS/EM indicating high resistance to cracking (Table 5.5). The addition of HPMC reduced this value significantly except for FPH04; however, TS/EM values remained high and acceptable. In the same line, RSE of films increased with the increase of the content in HPMC, being highest for FPH04 at 10.32 $N/mm^2 \cdot \%$, indicating crack resistance. Comparison of TI indicates that except for FPH01 which resulted to be the toughest formulation, TI of all other formulations varies in acceptable ranges (Table 5.5).

Table 5.5. Derived mechanical parameters calculated from conventional mechanical properties derived from a stress vs. strain plot. Results are represented as the mean and standard deviation in parenthesis.

Formulation	TS:EM $\%^{-1}$		Relative Surface Energy $N/mm^2 \cdot \%$		Toughness index $N/mm^2 \cdot \%$	
FPH01	5.69	$(1.94)^{i,ii,iii}$	4.59	$(1.06)^{a}$	216.51	$(21.32)^{i,ii,iii,iv,v}$
FPH02	3.36	$(0.32)^{i}$	4.69	$(0.63)^{a}$	92.08	$(15.55)^{i}$
FPH03	3.34	$(0.22)^{ii}$	8.62	$(0.77)^{b}$	88.10	$(22.67)^{ii}$
FPH04	4.11	(0.50)	10.32	$(1.93)^{b}$	60.32	$(15.70)^{iii,vi}$
FPH05	3.88	(0.50)	1.11	$(0.08)^{c}$	89.91	$(17.96)^{iv}$
FPH06	2.77	$(0.29)^{iii}$	1.75	$(0.08)^{c}$	106.33	$(20.12)^{v,vi}$

i - vi: Among parameters, statistically significant differences indicated in pairs of roman numerals ($p<0.05$). a – c: Among parameters, non-significant differences are indicated in pairs of letters.

5.4.2.2. Lys release and kinetics study

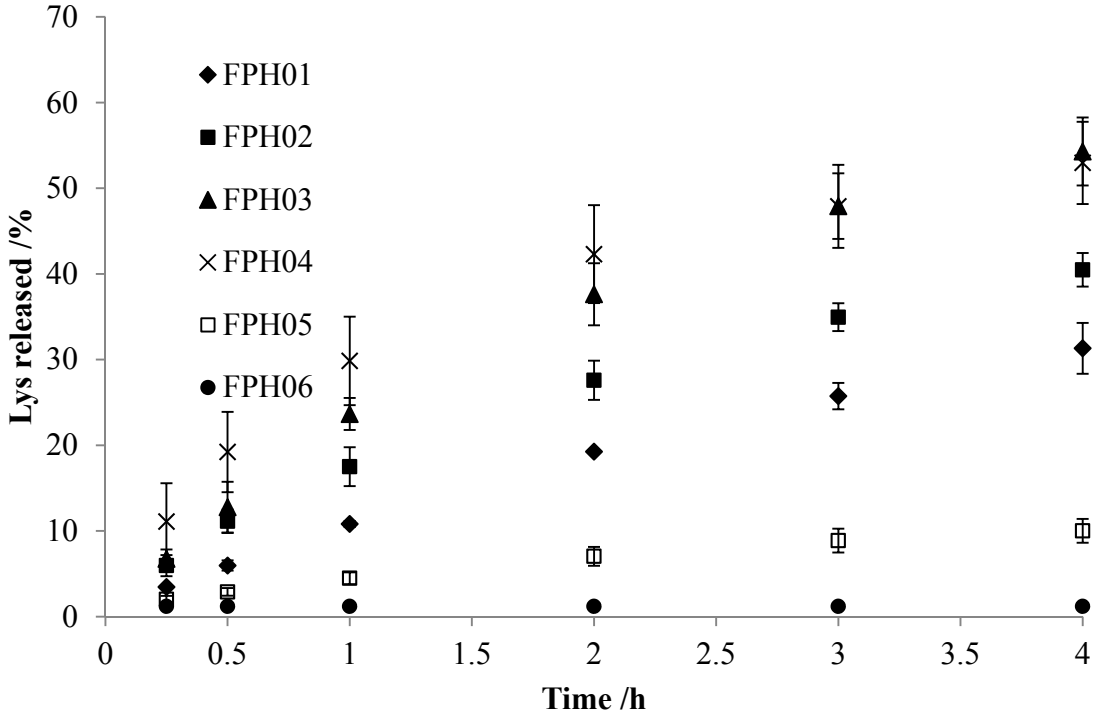

Figure 5.6. Lys release profiles from particle-containing films FPH01 (♦), FPH02 (■), FPH03 (▲), FPH04 (×), FPH05 (□), and the control FPH06 (●).

Table 5.6. Differences among FPH series of formulations based on the similarity factor, f2. Release profiles are similar if f2 ≥ 50.

f2	FPH01	FPH02	FPH03	FPH04	FPH05	FPH06
FPH01	--	40.76	22.47	20.73	27.23	19.40
FPH02		--	34.46	31.64	18.11	12.65
FPH03			--	56.79	9.75	5.89
FPH04				--	9.00	5.29
FPH05					--	45.23
FPH06						--

From the drug release profiles we can observe an increase in the release rate and extent of release as the concentration of HPMC increased in the formulations (FPH01–FPH04, Figure 5.6). HPMC is a water swellable and erodible polymer that will dissolve from the dosage form; therefore, increasing concentrations of HPMC in formulations allow for domains in the film that will release Lys faster than ERL-rich domains. In accordance with the similarity value, f2 [49], FPH03 and FPH04 are the only formulations that render a similar Lys release profile (Table 5.6). Therefore, an increase in the HPMC content from 30 to 50% w/w of polymer did not elicit significant differences in the release profile. According to the Korsmeyer-Peppas model, even though FPH04 has a higher constant (k equals 0.2800 for FPH04 and 0.2255 for FPH03) contributing to faster release at earlier times, the higher exponential term of FPH03 (n equals 0.6604for FPH03 and 0.4875 for FPH04) allows for faster release at later times. Similar effects have been described before in films combining HPMC and ERL [50]. Among the various materials studied, Hassan et al. found that the combination of HPMC

and ERL resulted in a lower burst release (< 20% drug released in the first 15 minutes) and in formulations that only contained HPMC a more rapid release was found to be associated with the swellable soluble matrix that HPMC constitutes in water [51]. Another study conducted by Averineni et al. shows the effect of having increasing concentrations of HPMC in chitosan-containing film formulations [52]. Over a 210 minutes period of time, drug release was increased from 52.52% to 73.23% for the formulations containing the lowest and highest amount of HPMC.

In the Kormeyer-Peppas release kinetics model, n is the release exponent, and is an indicative of the drug release mechanism [34]. In the particular case of n equal to 0.5 the drug release mechanism is purely Fickian diffusion (the particular solution that constitutes the Higuchi model equation). When n equals 1 the equation describes a zero order release mechanism, and the region ranging from $0.5 < n < 1$ represents the so-called anomalous transport. The first order kinetics applies to dosage forms that normally contain water-soluble drugs and porous polymer matrices. In said systems, drug release is proportional to the amount of drug remaining inside; therefore, the rate of drug release decreases with time. In Table 5.7 we can observe that except for FPH04, all formulations exhibit an anomalous release of Lys. This is a consequence of systems that are water swellable, where drug release occurs by a combination of diffusion and case-II transport. In the case of FPH04, the release is more adequately modeled by the Higuchi model (evidenced by the higher R^2). This indicates that drug release in this system follows Fickian diffusion through the polymer matrix. In addition, all formulations are better adjusted to the first order kinetics model (according to the R^2). This model describes drug release from porous matrices, such as that formed in a water swollen polymethacrylate

film, having a water soluble drug, such as the Lys-containing particles. In this system, drug release is proportional to the amount of drug remaining in the interior of the dosage form [35]. From the release profile we can also observe that when Lys was added to the film formulation as a solid solution very little release was achieved over the 4 hour period of time. Molecules in solid solution are completely surrounded by the polymeric matrix and a higher number of interactions between polymer and Lys can be achieved. This results in a very slow release over the time period (below LOQ).

Table 5.7. Model parameters and adjusted R^2 values for the FPH series of formulations.

Formulation	Korsmeyer-Peppas $Q = k \times t^n$			Higuchi $Q = k \times t^{0.5}$		First order $Q = k \times (1 - e^{-nt})$		
	k	N	Adj R^2	k	Adj R^2	k	n	Adj R^2
FPH01	0.1092	0.7702	0.9980	0.1407	0.9391	0.4956	0.2474	0.9995
FPH02	0.1728	0.6287	0.9955	0.1943	0.9791	0.4702	0.4672	0.9965
FPH03	0.2255	0.6604	0.9874	0.2612	0.9659	0.6579	0.4335	0.9998
FPH04	0.2800	0.4875	0.9751	0.2769	0.9881	0.5340	0.8316	0.9966
FPH05	0.0457	0.5837	0.9961	0.0492	0.9894	0.1114	0.5380	0.9911

5.4.2.3. Remaining Lys activity after film manufacture

After release for 24 hours in dissolution media activity of the Lys released was evaluated to measure any decrease in activity as an indicator of enzyme stability. As depicted in Figure 5.7, Lys remaining activity was excellent for all the formulations studied revealing that the processing of the manufactured particles into films for buccal delivery did not render the enzyme inactive. As shown above in the characterization of nanoparticles, enzyme activity is not compromised during the manufacturing process, and

the results obtained in this section show that further processing into polymeric films does not render the enzyme inactive. FPH05 exhibited a slightly lower activity which we believe was due to partial release of Lys over the 24 hours period (data not shown).

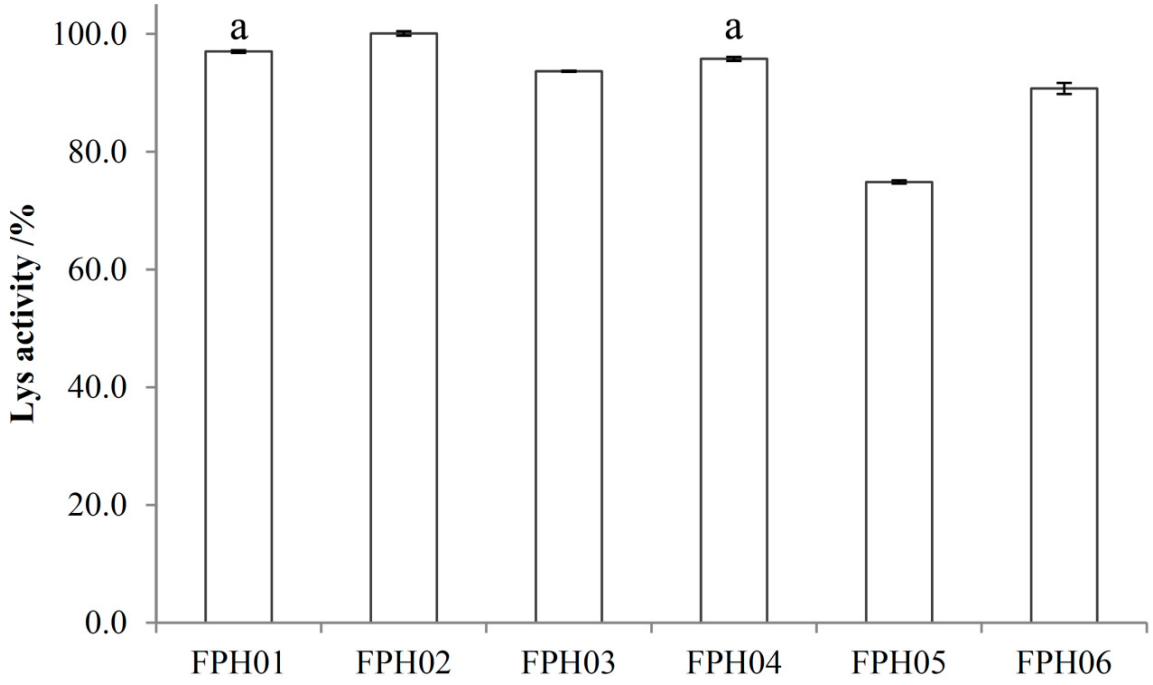

Figure 5.7. Lys relative activity (□) obtained from infinity release studies from film formulations. a: non-significant difference is indicated in pair of letters ($p>0.05$).

5.5. CONCLUSIONS

We have successfully developed mucoadhesive films for the delivery of Lys as a protein model. Lys was incorporated in the films as nanoparticles obtained by an antisolvent co-precipitation process. By controlling the pH and increasing the loading we were able to optimize conditions previously described. The new conditions for the method of manufacture allowed for the production of small and narrowly distributed particle with high yield and excellent remaining activity. Particles from formulation SPH02 were utilized in the manufacture of films for buccal delivery. All films containing Lys–coated nanoparticles had acceptable mechanical properties and Eudragit RL was shown to have excellent mucoadhesive properties. Additionally, films were able to sustain the release of Lys over 4 hours, modulated by the use of HPMC. Finally, we were able to achieve excellent enzyme activity maintained in films containing Eudragit RL. These films served as a preliminary model for their use with more therapeutically relevant macromolecules, such as insulin. The combination of the new particle manufacturing process and the use of films as solid delivery devices is depicted in the next chapter.

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6. Development of Films of Insulin-Coated Nanoparticles for use in Buccal Delivery

ABSTRACT

The goal of this investigation was to develop films containing insulin (Ins) in particulate form and assay its performance. The nanoparticle manufacturing process previously described was adapted to manufacture Ins-coated nanoparticles (ICNP). Particles were characterized for size, zeta potential, and yield after manufacture. ICNP were embedded in films utilizing Eudragit® RLPO (ERL) and its combination with HPMC by a solvent casting process. Films were characterized for morphology, mechanical properties, mucoadhesion, and Ins release. Furthermore, Ins permeation was evaluated using a human buccal mucose tridimensional model. ICNP were successfully obtained by our antisolvent co-precipitation manufacturing process. Particles adopted the typical flake-like morphology, and the batch containing 40% w/w Ins rendered 323±8 nm particles with a high zeta potential of 32.4±0.8 mV indicating good suspension stability. High yields (around 100%) were obtained after manufacture and the Ins content was retained after one month storage in an ongoing stability study. ICNP-embedded films using ERL as the polymer matrix presented with excellent mechanical properties, mucoadhesion, and release properties. ERL-HPMC-Ins films yielded more brittle films with high variability among the cast surfaces that translated into high variability of content, release, and permeation. Permeation enhancement was observed for both formulations when compared with a control Ins solution. Films containing ICNP were successfully developed with high yields of Ins after manufacture. The formulation containing ERL was found to be more effective in terms of film performance and

permeation enhancement of Ins through a human buccal mucosa model compared to the combination of ERL with HPMC.

6.1. INTRODUCTION

Protein and peptide therapeutics is a rapidly growing field in the pharmaceutical industry [1]. Due to specificity, which characterizes the action of proteins or peptides, only a relatively low dose is generally needed to obtain a therapeutic effect, making them ideal therapeutic agents and excellent alternatives for developing new medicines. However, it is widely reported in the literature that the efficacious delivery of these therapeutic agents can become the determinant factor for a product under development [2,3]. Therefore, in order to achieve the real potential of protein and peptide therapeutics, effective and smart delivery platforms and strategies, to overcome the formulation and delivery challenges, need to be developed.

More specifically, insulin (Ins) is the main therapeutic agent for the treatment of insulin-dependent diabetes type I, and many non-insulin-dependent diabetes type II patients [4]. Moreover, since Ins is usually associated with long term therapies, its delivery via injections impose several drawbacks in patient acceptance. For example, non-Ins dependent patients tend to delay the start of their Ins therapy when prescribed and one of the factors associated with this phenomenon, known as 'psychological insulin resistance', is the patient perception of the Ins injection or needle anxiety [5,6]. This has resulted in many attempts to develop different routes of administration, giving mixed results in the literature. The oral route has been widely explored and has been reviewed previously [7,8]. Many challenges are involved in the development of peptide formulations delivered through the oral route, such as gastric pH instability, proteolytic enzyme content in the upper GI tract, insufficient permeation and bioavailability. Very

limited commercial success has been achieved, as evidenced by the lack of products in the market [9].

Alternative routes of administration that have been investigated more recently in order to avoid the drawbacks of the GI tract [10,11] are the pulmonary, nasal, transdermal, and buccal routes. Of these, the buccal route has a number of advantages for the delivery Ins, such as the avoidance of the GI tract, and the associated advantages of this (avoidance of the acid-catalyzed degradation in the stomach, enzyme degradation in the intestine, first-pass metabolism, and low permeability). Additionally, the absence of a keratinized layer would result in improved absorption compared to keratinized mucosa. Further advantages associated with the buccal route include: a relatively low enzyme content compared with the intestinal content, a good supply of vascular and lymphatic drainage, ease of administration, and long cellular turnover (5-6 days) which may facilitate long term delivery in retentive dosage forms [12]. In order to formulate proteins or peptides we have previously reported on the development of films as buccal delivery dosage forms [13]. The most conventional approach for film manufacture is the combination of drug and excipients in solution followed by casting in a mold and drying. However, in general the polymers utilized in formulations containing proteins are more hydrophobic in comparison to the hydrophilic nature of Ins.[14] This could potentially lead to separation and/or aggregation during storage, or in vivo, leading to possible instability.[15] Additionally, homogeneity in drug distribution (and particularly for Ins-containing films) has not been addressed thoroughly in the literature. Even though chitosan has been reported as an adequate candidate as a polymer vehicle for Ins, no mention of Ins uniformity makes hard to reach a conclusion in homogeneity [16]. More

recently, Giovino et al. (2012) have developed chitosan films for the buccal sustained delivery of Ins in PEG-b-PLA nanoparticles as a model for buccal macromolecular delivery [17]. Although adequate physico-mechanical properties were achieved, very high heterogeneity was revealed by the mechanical variables studied, such as: time to break, tensile strength, Young's modulus, and work done to break. This therefore raises concern over the tight control of manufacture necessary to prepare films with homogeneous Ins distribution, adequate physico-mechanical properties, high yield and retention of macromolecule activity.

Recent developments in enzyme immobilization in organic solvents have allowed the development of novel method for the manufacture of protein-coated nanoparticles (PCNP), as described in Chapter 4 of this dissertation. This method was based on an improvement of an antisolvent co-precipitation method that has been shown before to successfully immobilize nucleic acids [18], proteins [19], enzymes [20], and other particulate systems [21]. However, most of these investigations lead to particles in the range of 1-5 μm and higher. To guarantee physical stability of the films in terms of mechanical and also mucoadhesive properties, such large particles are undesired due to potential for aggregation and loss in active distribution homogeneity [22]. However, submicron sized and nanosized particles have been found to produce films that comply with adequate mechanical and mucoadhesive properties [22]. Here, the antisolvent co-precipitation method described in Chapter 5 was adapted for the manufacture of nanoparticles to obtain Insulin-coated nanoparticles (ICNP). These particles were then embedded in film formulations for buccal Ins delivery and studied for physicochemical

properties, release and permeation through a human buccal mucosa three dimensional model.

6.2. MATERIALS

D,L-valine (Val) and insulin (Ins) were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MS). Sorbitan monostearate (Span60) was obtained from Spectrum (Spectrum Chemical, New Brunswick, NJ). Eudragit RLPO (ERL) was kindly donated by Evonik (Evonik Industries, Darmstadt, Germany). Carbopol® 974P (C974P) and Noveon® AA-1 Polycarbophil (PCP) were donated by Lubrizol (Lubrizol Advanced Materials, Cleveland, OH). Hydroxypropyl methylcellulose (HPMC, Methocel E50 Premium LV) was donated from Colorcon (Colorcon, Harleysville, PA). Triethyl citrate (TEC; Vertellus Specialties Inc, Indianapolis, IN), mucin (Spectrum Chemical, New Brunswick, NJ), and Micrococcus lysodeikticus (Worthington Biochemical Corp., Lakewood, NJ) were purchased and used as received. Human buccal mucosa was obtained from Mattek (Mattek Corporation, Ashland, MA). HPLC-grade 2-propanol (IPA) was obtained from Fisher Scientific (Fisher Scientific, Fair Lawn, NJ) while deionized water was procured in house. All other chemicals used were of analytical or reagent grade.

6.3. METHODS

6.3.1. Insulin-coated nanoparticle (ICNP) manufacture

The manufacturing process was based on antisolvent co-precipitation and our approach has been recently published elsewhere [23]. First, Val was dissolved in acid phthalate buffer pH 2.2 for a concentration of 61.2 mg/mL (or 90% w/v of its saturation concentration). Two different formulations were then manufactured to contain 10% and 40% w/w of Ins based in solid content. By means of an Aeroneb Pro® vibrating mesh nebulizer (Aerogen, Galway, Ireland), the aqueous phase was then added to the antisolvent organic phase. As we have shown earlier [23], IPA as the antisolvent combined with Span 60 is the most effective antisolvent; therefore, a 0.008 mM Span 60 solution was utilized. Finally, during the addition of the aqueous phase high energy mixing is provided by means of a Branson Sonifier 450 probe sonicator (Branson Ultrasonics, Danbury, CT). After addition of the total volume of aqueous phase sonication is maintained for a further 20 minutes to further stop particle growth during the early stages of coagulation [23].

6.3.2. Particle sizing by dynamic light scattering

To determine the particle size of the slurries obtained in 6.3.1, a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcester, UK) was utilized. Mean particle size was obtained as a Z-average which corresponds to the intensity weighted mean hydrodynamic size measured by dynamic light scattering (DLS). Additionally, an estimate of the width of the distribution was obtained from the instrument as a polydispersity index.

Approximately 1 mL of IPA slurry was analyzed by DLS in 1cm path length disposable polystyrene cuvettes. A total of 3-5 determinations of 15-20 runs each were conducted.

6.3.3. Zeta–potential determination

Zeta-potentials (ZP) of slurries were obtained by laser Doppler micro-electrophoresis using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Worcester, UK). Approximately 1 mL of slurry was added to a polycarbonate capillary cell for determination of ZP. A total of 5 determinations of 14–20 runs each were conducted to obtain the average ZP of the slurries.

6.3.4. Ins quantification by RP-HPLC

Chromatography was performed using a Zorbax 300SB® C18 Rapid Resolution column (3.5 μm, 4.6 mm ID x 150 mm length) (Agilent Technologies, Santa Clara, CA).The mobile phase consisted of two solvents with different polarities: solvent A consisted of water, 5% v/v acetonitrile and 0.1% v/v trifluoroacetic acid, while solvent B consisted of acetonitrile, 5% v/v water, and 0.085% v/v trifluoroacetic acid. The mobile phase consisted initially of 10% v/v solvent B with a solvent gradient of 60% v/v solvent B in 16 minutes. The flow rate was set to 1 mL/min and temperature remained constant at 25 °C. The injection volume was 50 μL and the UV detector was set to 215 nm. For the determination of Ins content on the slurries, particles were separated by centrifugation at 12,000 rpm then dried overnight at room temperature with an air current. The solids were then dissolved in pH 2.2 buffer and quantified with the RP-HPLC method. To determine

the content of Ins in films, release of Ins was allowed to occur over 24 hours at 37 °C in an orbital shaker and then the media assayed for Ins content by RP-HPLC.

6.3.5. Preparation of Ins-containing films

Casting solutions were prepared by combining two organic solutions and cast overnight in PTFE molds. Acetone was used to dissolve or suspend the polymer combinations as depicted in Table 6.1. This solution was combined in a 4:6 acetone to IPA ratio with suitable amounts of slurries obtained in Section 6.3.1 to yield the final casting solution. After 24 hours, films were peeled off and stored in aluminum foil sachets in a dessicator until characterization.

Table 6.1. Film formulations studied to investigate drug release and uniformity of films containing Ins particles.

Formulation	Eudragit RL % w/w	HPMC % w/w	TEC % w/w
ERL-Ins	90	--	10
ERL-HPMC-Ins	45	45	10

6.3.6. Morphology of particles and films by SEM

A scanning electron microscope Quanta 650 FEG (SEM, FEI Company, Hillsboro, OR) was used for imaging and ultrastructure analysis of both particles and particle-containing films. For imaging ICNP, droplets of IPA slurries obtained in Section 6.3.1 were deposited onto SEM stubs and allowed to dry on carbon tape before coating of the particles. For the imagining of films, cross-sections were obtained by a freeze fracture

method to ensure clean-cut edges and to avoid plastic deformation (often resulting from mechanical cutting) [22]. Fragments of films were flash frozen by submerging in liquid nitrogen and thus cracked by freezing. Pieces of the films were fixed on aluminum stubs by means of conductive carbon tape for coating. Coating was performed using a 208 HR Cressington sputter coater (Cressington Scientific Instruments Ltd, Watford, UK) with Pt/Pd to a thickness of 10–12 nm in a high vacuum evaporator. To avoid structural deformation during imaging, the electron beam voltage was kept low at 2–3 kV [24].

6.3.7. Mucoadhesive and mechanical properties of films in vitro

Mucoadhesion tests were conducted using a TA.XTPlus texture analyzer (Stable Micro Systems, Godalming, UK) equipped with a 5 Kg load cell. Briefly, films were held in the horizontal position and 5 µL of model mucus (a freshly made 2% w/v mucin solution) was placed on top of the film. This amount is sufficient to mimic the thickness of the average saliva thickness [25]. A 7 mm diameter stainless steel cylindrical probe was attached to the mobile arm of the texture analyzer and it was brought in contact with the film and mucin solution, held at an applied force of 50 mN for 15 seconds and then withdrawn at a 0.5 mm/second rate. Mucoadhesive force (MAF) and work of adhesion (WoA) were obtained from the peak and the area under the curve in the force versus distance profile, respectively.

For the determination of mechanical properties, rectangular strips of 1 x 5 cm^2 were cut and 1 cm on each end was held between clamps attached to the texture analyzer, leaving a testing area of 1 x 3 cm^2. The upper clamp (connected to the mobile arm of the texture analyzer) was moved upwards at a rate of 0.5 mm/sec until film failure. Stress is

obtained from the force measurements obtained from the instrument divided by the cross-sectional area of the film, while strain is computed by dividing the increase in length by the initial film length. From the plot, the tensile strength (TS) and the elongation at break (EB) are obtained from the peak stress and the maximum strain, respectively, also represented by the following equations [26]:

$$Tensile\ strength\ (TS) = \frac{Peak\ stress}{Cross-sectional\ area\ of\ film}$$

$$Elongation\ at\ break\ (EB) = \frac{Increase\ in\ length\ at\ break}{Initial\ film\ length} \times 100$$

Additionally, the elastic modulus (EM) was obtained from the initial elastic deformation region in the stress vs. strain plot [27]. Since the rate of the mobile arm was constant during the test as well as for all different experiments, direct comparison of the slope in this region can be done. To further evaluate mechanical properties TS:EM ratio (TS:EM), relative surface energy (RSE), and toughness index (TI) were computed from the conventional mechanical parameters obtained from the plot as shown in the equations below [28]:

$$Tensile\ strength\ to\ modulus\ ratio = \frac{TS}{EM}$$

$$Relative\ surface\ energy\ (RSE) = \frac{TS^2}{2 \times EM}$$

$$Toughness\ index\ (TI) = \frac{2}{3} \times TS \times EB$$

6.3.8. Ins release and kinetics analysis

Ins release was performed on Franz diffusion cells and phosphate buffer pH 6.8 as media. To support the films and avoid solid disintegration into the receiving chamber a 0.1 μm nylon membrane filter was used (so as not to limit diffusion). Films were cut into 1.5 cm diameter circular samples (n=3) and allowed to release into the reservoir media for 4 hours. At intervals of 0.25, 0.5, 1, 2, 3, and 4 hours, 300 μL samples were withdrawn and replaced with fresh media. Ins content was determined with the method described in Section 6.3.4.

To investigate the mechanisms involved in the lysozyme release, kinetics models were compared to the release profiles. The Higuchi [29], Korsmeyer-Peppas [30], and first order kinetic models were used to fit the data and were compared on the basis of R^2 adjusted [31]. The evaluation of the drug transport mechanism was addressed in accordance with the Korsmeyer-Peppas model.

6.3.9. Ins permeation through a human buccal mucosa model

Three dimensional models of human model mucosa obtained by differentiation of normal human buccal keratinocytes were obtained for this study (Mattek Corporation, Ashland, MA). Tissues grown in inserts were equilibrated at 37 °C for one hour prior to

permeation experiments with 0.9 mL of PBS in 6-well plates. In this way only the basolateral side of the tissue was exposed to the medium. At 0.25, 0.5, 1, 2, 3, and 4 hours each insert was transferred to the next well containing 0.9 mL of fresh PBS to allow for further permeation of Ins. Ins permeated through into the basolateral solution was quantified by the method depicted in Section 6.3.4.

6.3.10. Statistical analysis

All statistical analyses were performed with the software Minitab Release 14® (Minitab Inc., State college, PA). T-tests were used when comparing two sets of data while one-way ANOVAs were used for multiple comparisons and Tukey's post hoc pairwise comparisons were performed to compare which results led to significant differences. Release kinetics were modeled using the DDSolver program [32] for Microsoft Excel®. For comparison of release profiles, the similarity factor (f2) was computed using the DDSolver program. f2 values greater than 50 indicate similarity between release profiles. All values are reported as the mean and standard deviation of the mean shown in parenthesis.

6.4. RESULTS AND DISCUSSION

6.4.1. Manufacture of insulin-containing nanoparticles by an antisolvent co-precipitation process

Previous findings by our group and others have demonstrated the use of the antisolvent precipitation process as a means to obtain multiparticulate systems with high

yield and activity. We have previously shown that both BSA and Lys nanosized particles can be obtained by a method that combines high energy mixing and a high surface of contact between the solvent and the antisolvent [23]. Here we have successfully obtained Insulin-coated nanoparticles (ICNP) by a similar method (Table 6.2). It was the use of a high content of the peptide that allowed for the smallest particles size (323±8 nm) and highest ZP (32.4±0.8 mV). The high value of ZP allowed for the slurry to remain in suspension for over two weeks compared to Ins 10% w/wthat sedimented after 48 hours.

Table 6.2. Particle size reported as z-average, polydispersity index, and zeta-potential of the Ins formulations investigated. Results are represented as the mean and standard deviation in parenthesis.

Formulation	Z-average /nm	Polidispersity index*	Zeta-potential /mV
Ins 10%	819 (48)	0.44 (0.14)	18.3 (0.3)
Ins 40%	323 (8)	0.42 (0.02)	32.4 (0.8)

*: except for polydispersity indices, all pairwise comparison were significantly different ($p<0.001$).

Particles adopted the typical flake-like shape observed before in particles [23] where the core is composed of Val (Figure 6.1). In typical antisolvent co-precipitation processes the particle shape is determined by the crystalline structure of the core material [21,33]. Murdan et al. have found that the use of L-glutamine as the crystalline core material in diphtheria toxoid-coated microparticles resulted in crystalline material in similar shape to the naked amino acid material processed by the same method. The use of diphtheria toxoid in the aqueous phase resulted in a particle size decrease down to less than 10 μm [33]. In our studies we found that the addition of Ins significantly decreased

particle size from 888±10 nm for pure Val nanoparticles [23] to 819±48 nm with 10% w/w Ins and further down to 323±8 nm with 40% Ins w/w. This has been previously described for the process in several occasions in the literature [18–20,34] and it is the result of the molecule precipitating on the surface of the core material acting as a stabilizer much like the effect of a surfactant. Kreiner et al. described the effect of subtilisin Carlsberg as a precipitation "poison" by occupying the surface of the crystals and impeding further particle growth [34].

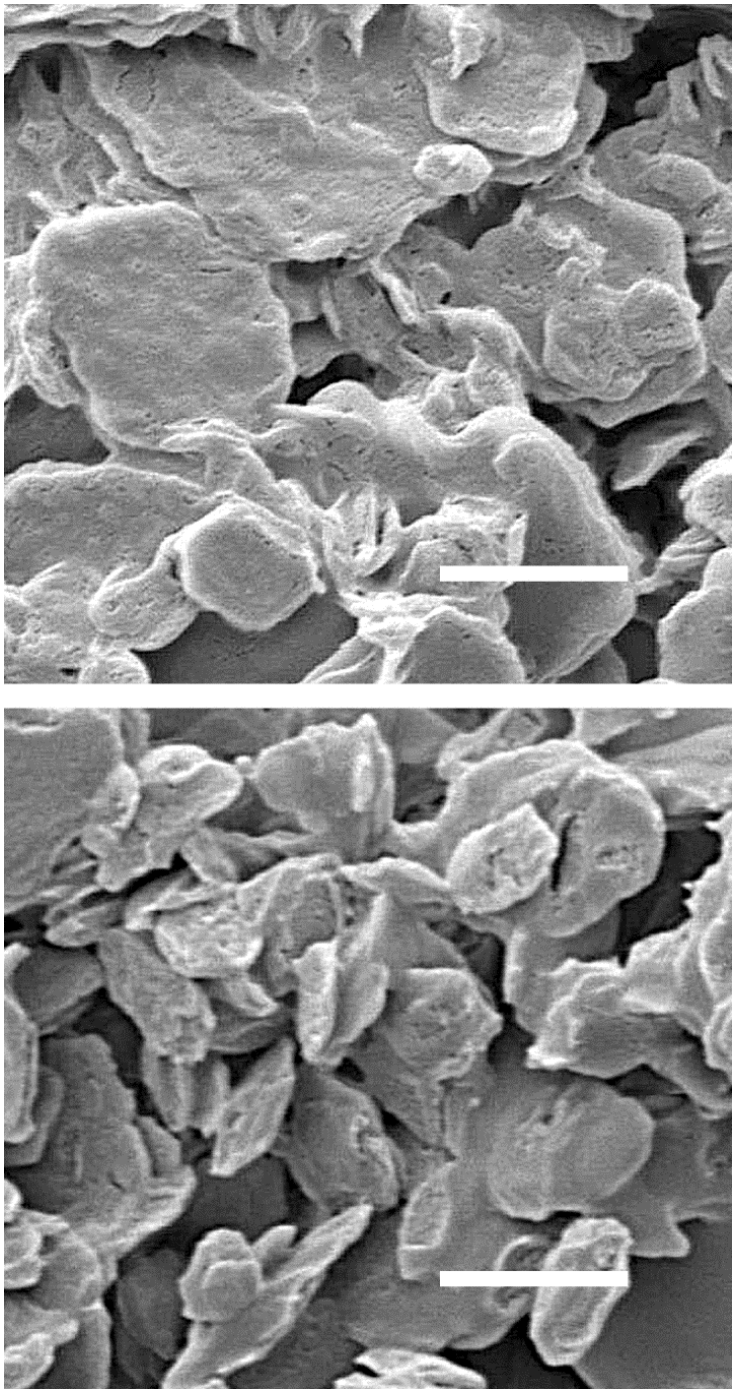

Figure 6.1. SEM micrographs of ICNP from Ins 10% w/w (top) and Ins 40% w/w (bottom). The bar represents 1 μm.

The antisolvent co-precipitation process has also been described as a process to render highly active and stable protein and peptide-containing particles. Here, even though Ins is subjected to high energy mixing via sonication, very high yields are achieved following manufacture (Figure 6.2). In general, exposure to organic solvent and high mixing energy can decrease the activity of labile molecules such as proteins and peptides by modifying their secondary and tertiary structures [35]. Nonetheless, the rapid dehydration that Ins molecules go through in the process described here allows for the maintenance of the molecular structure. Partridge et al. attributed this effect to the way that precipitation occurs [20]. During the precipitation process, molecules of the precipitant in their native aqueous form lose their solubilizing water molecules and are immobilized onto the surface of the core material crystals and retain this conformation after manufacture. Therefore, high yields and high activity can be achieved by using this process of nanoparticle manufacture. Similarly, high stability of Ins content in the particles manufactured were found after one month of storage under room conditions. The differences in Ins yield after one month of storage in room conditions (25 °C and 60 % relative humidity) were not statistically significant ($p>0.05$) and it was overall excellent over 90% for both formulations (Figure 6.2). We have found similar results during production of lysozyme-coated nanoparticles obtained by the same method after three months of storage (data not shown). Partridge et al. observed similar results in subtilisin Carlsberg-coated microcrystals stored at 60 °C for six months [19].

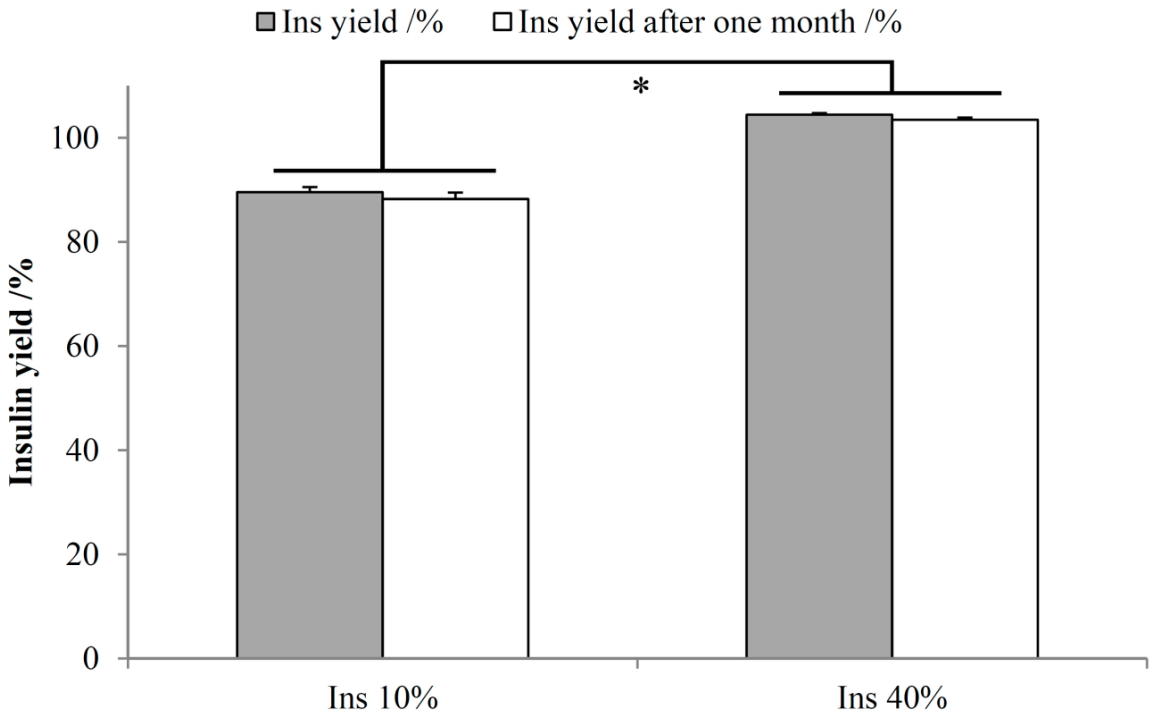

Figure 6.2. Ins yield after manufacture and stability after one month. *: Only between formulations the differences were significant ($p<0.05$). Yield decrease due to storage was not statistically significant ($p>0.05$).

6.4.2. Development and of films for buccal delivery of Ins

6.4.2.1. Morphology, mucoadhesion, and mechanical properties of films

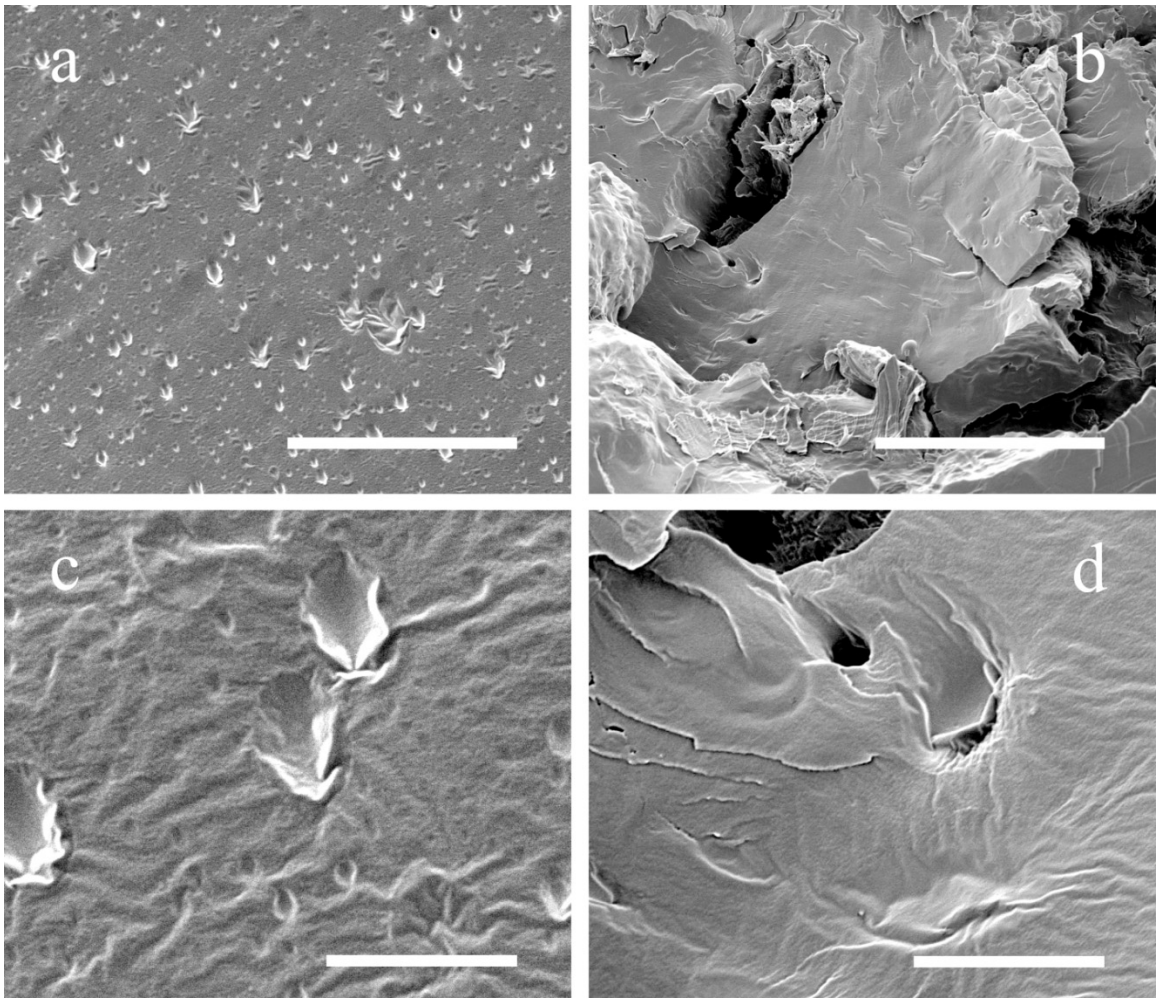

Figure 6.3. SEM micrographs of cross-sections of films obtained by freeze-fracture. (a and c) ERL-Ins and (b and d) ERL-HPMC-Ins films. Bar on top micrographs represents 20 μm while bar on bottom micrographs represents 5 μm.

Successful manufacture of films was achieved with both polymers utilized. ERL films appeared homogeneous to the eye and inspection of cross-sections obtained by freeze fracture revealed a homogeneous distributions of what appears to be flakes of Ins-coated nanoparticles (Figure 6.3c). Alternatively, films manufactured by combining ERL

and HPMC present with a more discontinuous matrix due to the distribution of HPMC throughout ERL. However, distinct domains reveal the presence of similar flake-like particles in the polymer matrix, similarly to the evidence found in ERL films (Figure 6.3d). These findings are in agreement with what we have previously found in Lys-coated nanoparticle-containing films [13]. Both ERL and ERS were found to exhibit a homogeneous appearance in cross-sections and films combined with HPMC revealed distribution of HPMC-rich domains [13].

ERL was found to be an excellent mucoadhesive material regardless of exhibiting a slightly lower MAF compared to typical mucoadhesive materials such as C974P and PCP. As previously described, WoA of films containing PCNP was found to be notably higher than that observed in typical mucoadhesives [13]. The high solubility of the ICNP homogeneously distributed among ERL films allows for water penetration to be faster into the polymer matrix. This results in a faster softening effect on the polymer chains and an increase in flexibility of the film upon probe withdrawal. Finally upon probe withdrawal, the film can deform to a higher extent increasing the distance of the applied force resulting in an increase on WoA. In mucoadhesion theories of diffusion [36] and water movement [37], water plays a most important role in establishing the mucoadhesive bond. Water interacts with the polymer chains and softens the polymer matrix allowing for entanglement of domains of mucin molecules and polymer chains. Additionally, Nafee et al. have demonstrated that the presence of particulate material disrupts the continuum of the polymer matrix allowing for more free space for polymer movement and entanglement to happen [38]. A decrease in mucoadhesive properties by the addition of HPMC to an ERL polymer matrix has been previously described [13], and

is a consequence of slower wetting acting to lower mucoadhesion. A lower extent of mucoadhesion than that observed in films containing either C974P and PCP has also been reported for HPMC [39,40]. Wong et al. have found a similar trend to a decrease in mucoadhesive properties by combining HPMC with polymethacrylates [41].

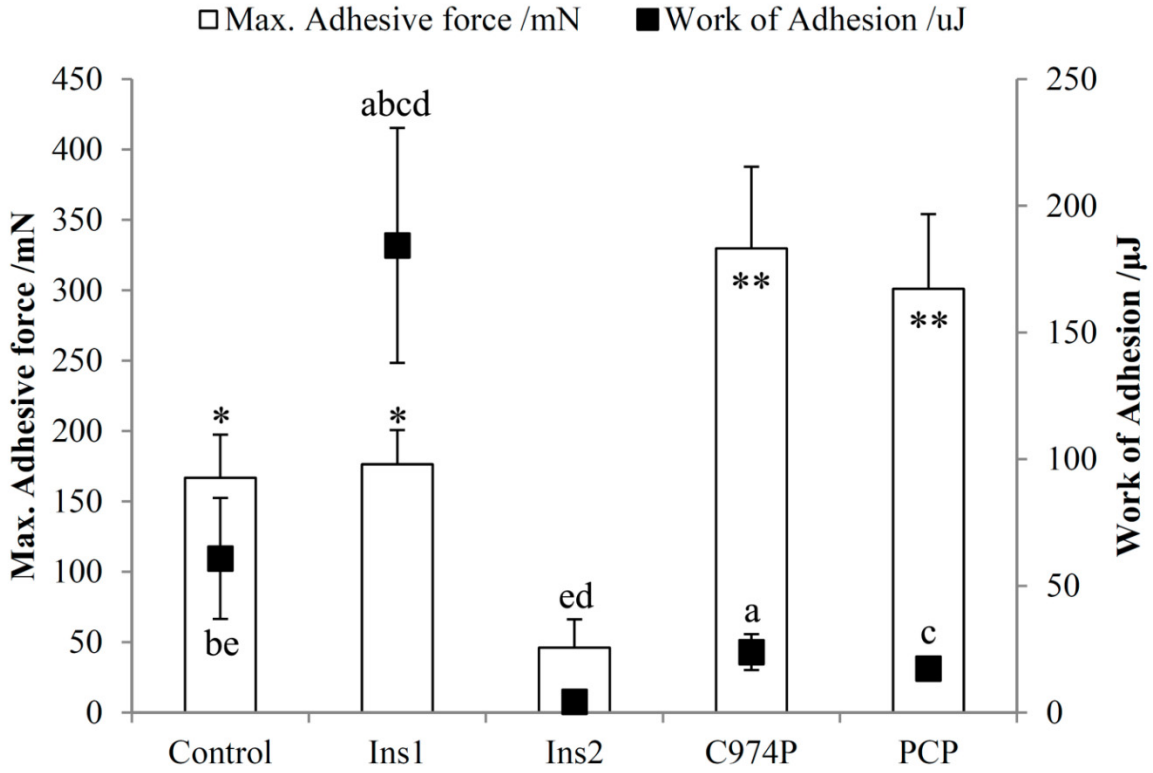

Figure 6.4. Mucoadhesive properties of Ins-loaded films and typical mucoadhesive materials. Maximum adhesion force (□) MAF, with non-significant differences indicated with asterisks (*, **, $p>0.05$); and Work of adhesion (■) WoA, with significant differences indicated in pairs of letters (a-e, $p<0.01$).

Not only is mucoadhesion needed for films to be ideally placed in contact with the buccal mucosa, but strength and flexibility as determined by the mechanical properties of the films, are also paramount to withstand stress that originates from normal mouth

mechanical activities [42]. Furthermore, for handling and shipping of films as dosage forms, mechanical properties such as elongation, strength, and toughness are needed. In Table 6.3 we can observe that the inclusion of particles in the polymer matrix decreases strength, toughness, but increase elongation slightly compared to pure ERL films plasticized to the same extent with TEC. The disruption of the polymer continuum results in slightly softer acceptable films as solid dosage forms [42,43]. The addition of HPMC drastically decreased elongation and highly increased strength which is associated with a decrease in TI. Films low in TI become more brittle [28] which is further corroborated by the increased RSE observed [43]. RSE is used to estimate crack resistance and is approximated from the surface energy of the film, thus a high RSE indicates brittleness in films.

Table 6.3. Direct and derived mechanical properties for Ins-containing films. Results are represented as the mean and standard deviation in parenthesis.

Formulation	Tensile strength N/mm^2	Elongation at break %	Elastic modulus $N/mm^2/\%$
Control	1.455 (0.120)*	217.7 (30.5)	0.513 (0.062)
ERL-Ins	0.514 (0.010)*	277.0 (14.3)	0.099 (0.009)
ERL-HPMC-Ins	5.500 (0.945)	14.6 (3.5)	1.337 (0.275)
	TS:EM $\%^{-1}$	Relative Surface Energy $N/mm^2 \cdot \%$	Toughness index $N/mm^2 \cdot \%$
Control	2.86 (0.34)	2.09 (0.37)*	212.69 (44.34)
ERL-Ins	5.21 (0.52)	1.34 (0.14)*	94.83 (3.90)*
ERL-HPMC-Ins	4.14 (0.21)	11.33 (1.65)	52.68 (10.18)*

*: indicated pairs of data among parameters were not found to be statistically different ($p>0.05$).

6.4.2.2. Ins yield, release and kinetics

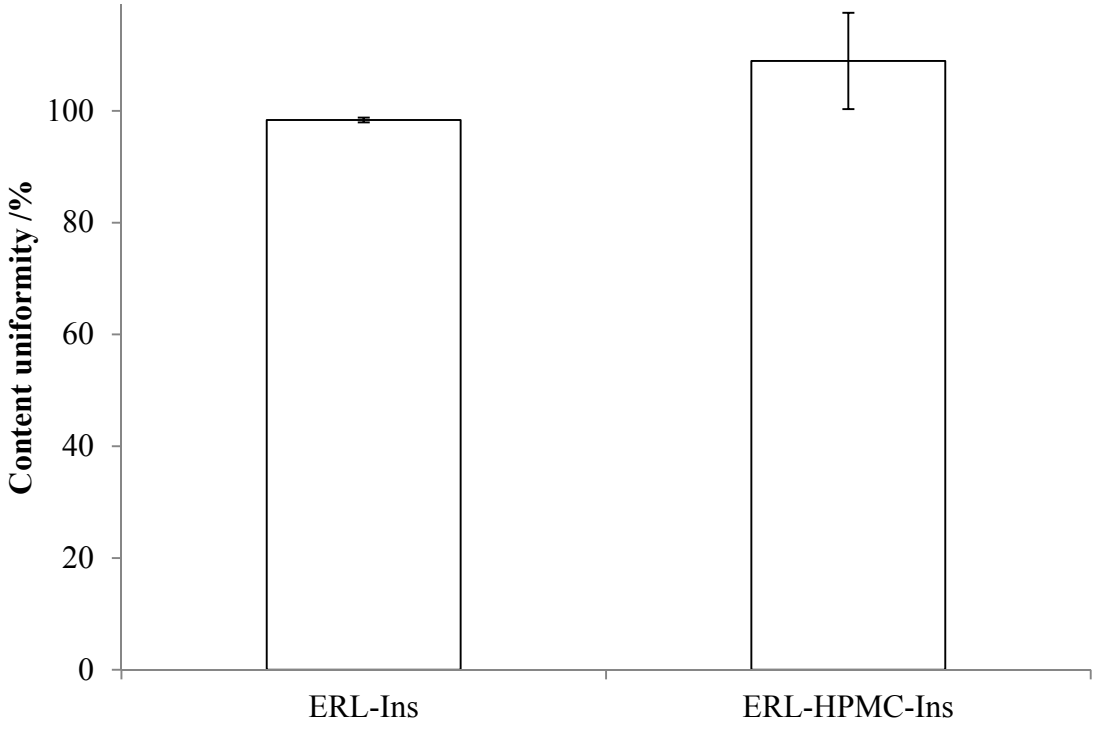

Figure 6.5. Ins content uniformity after release over a 24 hours period of time for ERL-Ins and ERL-HPMC-Ins films.

To establish the yield of Ins-loaded films, release was allowed over 24 hours to quantify the content of Ins. Figure 6.5 shows the yields obtained for both formulations by comparing the assayed amount of insulin with the theoretical amount. It can be observed that practically all the estimated amount of Ins was contained in the films. However, a clear difference in uniformity can be depicted. ERL-HPMC-Ins films in particular exhibited a relative standard deviation of about 9%, which was much higher than the

0.4% obtained for ERL-Ins films. This also translated into high variability of release as observed in Figure 6.6.

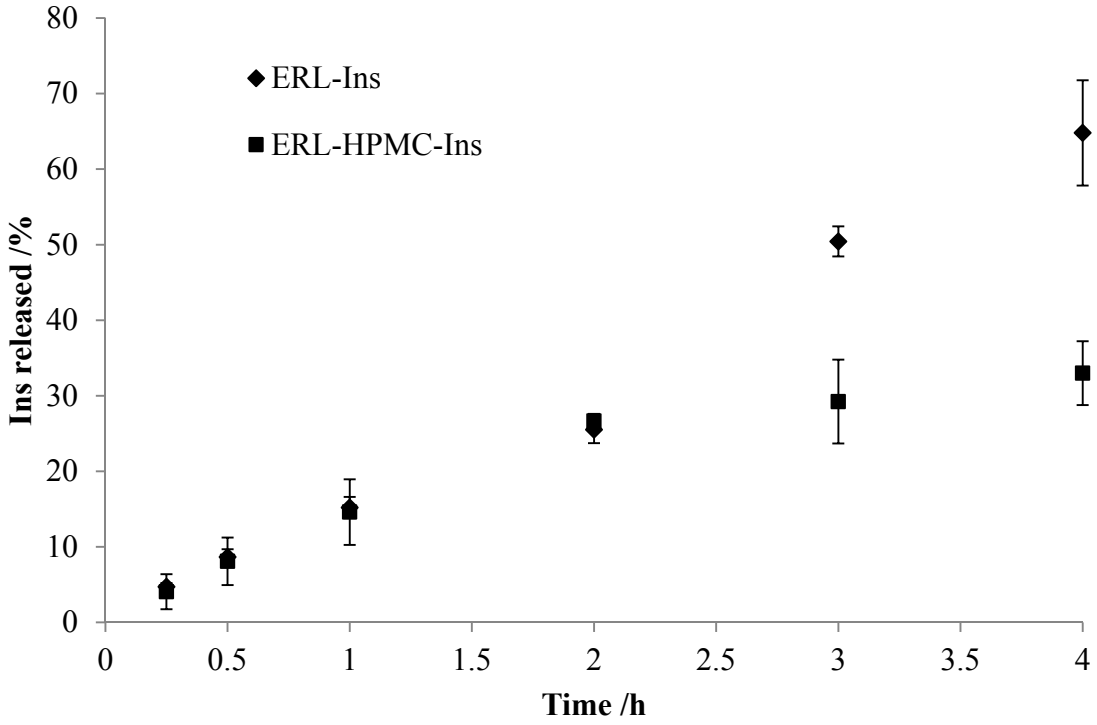

Figure 6.6. Ins release studies for ERL-Ins (♦) and ERL-HPMC-Ins (■) formulations.

Figure 6.6 reveals that both formulations presented with similar release at early time points, but at later times ERL-Ins films released to a higher extent of about 65% over 4 hours. This resulted in a similarity factor (f2) lower than 50 (41.9) indicating that the release profiles are different. The higher extent of variability observed for ERL-HPMC-Ins films can be attributed to the presence of HPMC which is responsible for an increase in heterogeneity of the polymer matrix. It has been described in the literature

that HPMC shows a faster release compared to that observed for ERL and this is attributed to a higher extent on swelling upon hydration [44,45]. Similar effects have been observed in the addition of HPMC to chitosan buccal films where the extent of release was increased from 52.5% to 73.2% over a 210 minute period [46]. Therefore, in these studies an increase in variability was observed due to the higher release of HPMC-rich domains that contribute to heterogeneity. Signs of heterogeneity can also be observed in cross-sections obtained by freeze fracture in Figure 6.3.

Both release profiles are best explained by the Korsmeyer-Peppas equation by inspection of the R^2 values obtained in Table 6.4. The Korsmeyer-Peppas release model classifies release profiles according to the value of the exponent term n. Briefly, when n equals 0.5 the drug release mechanism is purely Fickian diffusion (in this particular case the KP model equates to the Higuchi model too). When n equals 1 the equation turns into a zero order release kinetics that is usually associated to water-soluble drugs contained in porous polymer matrices. And the region $0.5 < n < 1$ is referred to as an anomalous transport release mechanism. The n value of ERL-Ins films reveals release profile very close to a zero order release mechanism indicating that drug transport is mostly due to solubilization of Ins and diffusion out of the porous ERL matrix. Conversely, an n value of 0.631 observed for ERL-HPMC-Ins films indicates that a combination of phenomena turns the drug release mechanism in an anomalous transport. This is possibly due to the distribution of the drug in ERL-rich and HPMC-rich domains from which drug is released at different rates according to the release mechanism of each domain. HPMC is a highly water swellable and soluble polymer that releases drug by diffusion and erosion of the matrix[45] resulting normally in faster release profiles than those observed for either

ERL and ERS [13]. This is also in agreement with the higher variability observed in ERL-HPMC-films where two mechanisms of release are occurring at all times (Figure 6.6).

Table 6.4. Model parameters and adjusted R^2 values for the insulin-containing films.

Formulation	Korsmeyer-Peppas $Q = k \times t^n$			Higuchi $Q = k \times t^{0.5}$		First order $Q = 100 \times (1 - e^{-nt})$	
	k	N	Adj R^2	k	Adj R^2	n	Adj R^2
ERL-Ins	14.311	1.086	0.9885	25.851	0.8378	0.217	0.9569
ERL-HPMC-Ins	14.669	0.631	0.9595	16.436	0.9456	0.119	0.9211

6.4.2.3. Ins diffusion through a human buccal model

EpiOral® is a buccal mucosa model developed by culture of primary buccal keratinocytes on a fibroblast containing collagen-based matrix. These cells differentiate into a three dimensional model of about 8-11 cells layers thick. In comparison with other in vitro human buccal mucosa models it has been described as being the histologically closest to normal mucosa together with full-thickness oral mucosa [47].

Studies on the permeation of Ins through a human buccal mucosa revealed a similar trend in permeation compared to the extent of release and variability among formulations. Figure 6.7 shows the cumulative permeated Ins as a function of time through the buccal mucosa. Compared to the control group, both formulations successfully allowed for the penetration of Ins through the mucosa and enhanced the permeation extent. This effect of enhancement we believe is similar to what has been

described for chitosan [48]. Chitosan is another mucoadhesive material containing positively charged groups in its structure which allows for its mucoadhesion, much like the ERL. The permeation enhancement effect is attributed to the mucoadhesive material acting by increasing the retention time in contact with the buccal mucosa and creating a deposition of active material in the vicinity of the cell barrier, creating a high concentration gradient directing the release of active in the direction of the buccal mucosa [49]. This also explains the differences in extent of permeation enhancement between the two formulations. ERL-Ins releases Ins faster increasing the concentration gradient resulting is faster penetration of Ins compared to that of ERL-HPMC-Ins. Even though the difference in enhancement is clear from Figure 6.7, no statistical differences were observed for either Ins flux or lag time (Table 6.5). This is a consequence of the very high variability of permeation observed for ERL-HPMC-Ins films having domains that permeate Ins similarly to ERL-Ins, as well as HPMC-rich domains responsible for slower release of Ins. Regardless of this, permeation of Ins is greatly enhanced by the use of ERL and films as solid dosage forms achieving a flux of 0.34±0.05 μg/hr/cm^2 and very little lag time at 7.8 minutes.

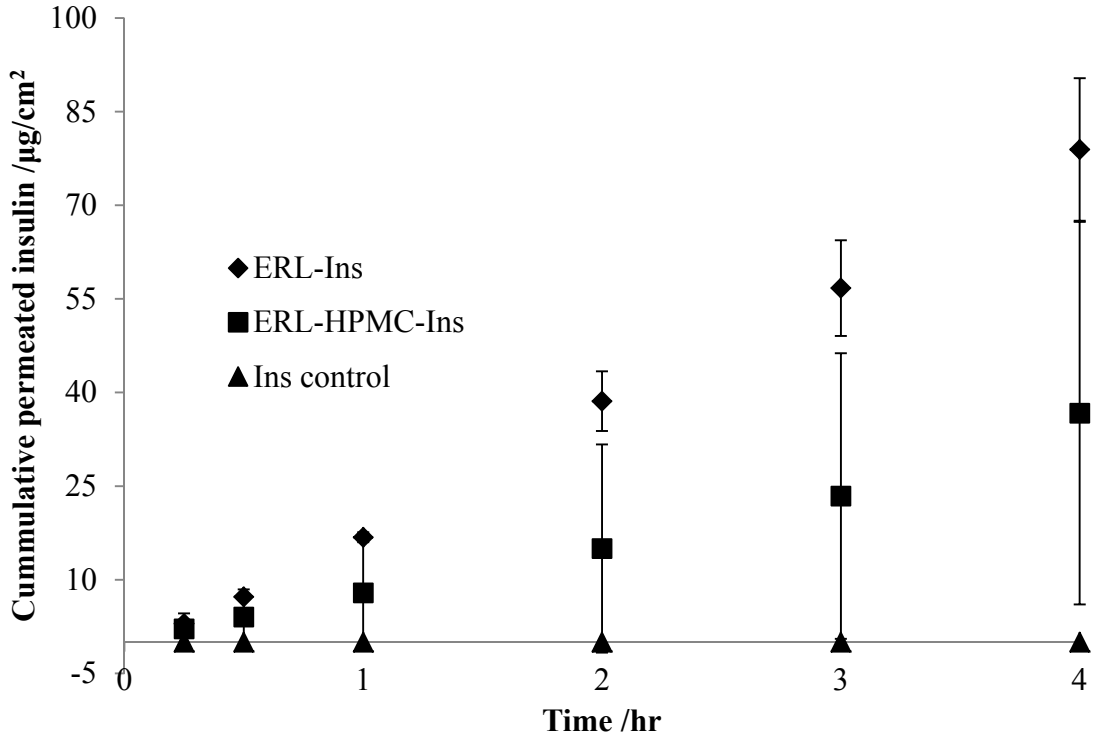

Figure 6.7. Cummulative Ins permeation of ERL-Ins (♦), ERL-HPMC-Ins (■) formulations, and a control solution of insulin PBS (▲). Permeation was performed using a human tridimensional buccal mucosa model.

Table 6.5. Permeation flux and lag time observed for Ins-containing films. Results are represented as the mean and standard deviation in parenthesis.

Formulation	Flux µg/hr/cm^2	Lag time min
ERL-Ins	0.34 (0.05)	7.81 (2.66)
ERL-HPMC-Ins	0.13 (0.13)	5.14 (6.34)

6.5. CONCLUSIONS

We have shown that the antisolvent co-precipitation method of manufacture of nanoparticles is suitable for obtaining ICNP with great yield and stability upon storage. Particles aligned to the typical flake-like structure of the core material, Val, and the use of Ins at higher loadings further decreased particle size.

ICNP were then successfully formulated into mucoadhesive films for buccal delivery of Ins. Particularly, ERL-Ins films were found to perform best in comparison with ERL-HPMC-Ins films in most variables studied. Homogeneity was higher in ERL-films which resulted in a tighter control over physic-mechanical properties as well as release and permeation. Higher extent of permeation enhancement by the use of ERL was observed in ERL-Ins films compared to an Ins control solution. Studies to test these films in an animal model are warranted.

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7. Conclusions

Films for buccal delivery of insulin (Ins) were successfully developed. Optimized film formulations exhibited excellent mechanical and mucoadhesive properties when loaded with protein-coated nanoparticles. Through an investigation of caffeine particles in films the existence of a particle size cutoff above which the physical properties of films are compromised has been described in this work. A direct consequence of higher particle size is non-uniform agglomeration throughout the surface of the film resulting in high variability in several properties of the film including content uniformity and drug release. In addition, the polymethacrylate Eudragit® RLPO (ERL) was found to be an excellent mucoadhesive polymer. Typically regarded in the literature as a hydrophobic material, no previous applications as a mucoadhesive in films for buccal delivery have been reported. The best performing ERL formulation achieved very high work of adhesion (WoA) of 118.9 μJ, which corresponded to about 5-6 times higher than the WoA observed for commonly used mucoadhesives such as Carbopol 974P (C974P, 23.9 μJ) and polycarbophil (PCP, 17.4 μJ).

Proteins, peptides, and macromolecules in general, are known to be labile molecules that could be rendered inactive by a variety of processes. It has been reported that adding Ins into polymer matrices as a solid solution results in loss of activity. In addition, contact of proteins or peptides with organic solvents can also hinder the molecular structure and thus their stability. Chapter 4 described a method for the manufacture of protein-coated particles with high yields but more importantly, high activity after manufacture even when the process involves the use of organic solvents. The antisolvent co-precipitation process consists of precipitating macromolecules on the

surface of growing core crystals typically composed of an amino acid. The aqueous phase contained both the core forming material and the protein or peptide of interest and this solution was then added onto an organic solvent miscible with water. The addition of the aqueous phase into the organic phase starts a rapid dehydration process that triggers the precipitation of the core material first (due to its high concentration near saturation) and of the protein. This work described a novel method of manufacture based on antisolvent co-precipitation adapted to render nanoparticles. Investigations on the processing and formulation parameters governing this method revealed that a combination of high energy mixing by means of a probe sonicator, the use of Span 60 as a stabilizing surfactant at its critical micelle concentration, and the addition of the aqueous phase with a high surface area for interaction with the organic phase using nebulization resulted in submicron sized and nanosized protein-coated particles for all models. These particles were found to exhibit excellent yields and activity after manufacture regardless of the macromolecule exposure to organic solvents. This is a consequence of the rapid dehydration that takes place upon combination of both phases. The protein or peptide of interest loses their solubilizing water molecules and it immobilized onto the surface of the growing core crystals in a native form. This allows for high stability during the process that further translated into high activity after manufacture.

To render films with adequate physical properties containing particulate material, submicron sized particles were found to be needed. The work on protein-coated particles described in Chapter 4 and further work on the effect of pH over particle size, yield, and activity allowed for obtaining optimized protein-coated nanoparticles (PCNP) based on the antisolvent co-precipitation process. These particles were embedded in platform film

formulations that are intended to be suitable for the buccal delivery of proteins, which was inferred in one respect by the high yield and activity they exhibited. Successful film formulations could be obtained by adding slurries of nanoparticles on solutions containing mucoadhesive polymers and casting them as described in Chapter 3. Excellent mucoadhesive properties were achieved in films containing ERL embedded with PCNP. The presence of the water-soluble nanoparticles enhanced mucoadhesion of ERL films even further due to an increase in water migration into the polymer matrix. The release of Lys could be tuned with the use of HPMC as a water-swellable and soluble material. Over four hours of active release could be controlled by adjusting formulation parameters, and the Lys remained fully active during this period of time.

Finally, all the work completed in earlier Chapters of this dissertation led to the development of Ins-loaded films. These films were obtained by first adapting the method of manufacture of nanoparticles for the manufacture of Ins-coated nanoparticles (ICNP) and then embedding said particles on polymer matrices. High loads of Ins (40 % w/w) could be achieved in ICNP which resulted in an even greater decrease in particle size with excellent yield. The higher loading of Ins limited the growth of particles by acting like a stabilizing surfactant on the surface of nanoparticles. ERL was again found to be the best performing material in terms of physicochemical properties. Ins release from ERL-Ins films was found to achieve greater and more uniform concentrations than those observed for ERL-HPMC-Ins films. Additionally, *in vitro* permeation studies conducted on a three dimensional human buccal model revealed an enhancement on permeability when Ins was loaded on films. This was due to the higher concentration gradient that is achieved by loading the active in films and thus steering the release in the direction of the

buccal mucosa. Thus, Ins-loaded films were developed and successfully delivered the active through a buccal mucosa model while maintaining excellent physicochemical properties.

The research conducted for this dissertation was aimed at developing a suitable dosage form for successful delivery of Ins through a buccal mucosa model. It was found that films are suitable candidates as a solid dosage form with great potential for being adapted to the delivery objectives sought. Particulate material containing macromolecules in the form of protein-coated nanoparticles were found to improve the physicochemical characteristics of films and warranted high yields and activity of the loaded active. The *in vitro* demonstration of Ins permeation makes an *in vivo* study the logical next step in this investigation. Due to the similarity to the human mucosa and comparative convenience to other models, the mini pig would be the animal of choice for pharmacokinetic and pharmacodynamics studies. Rodents, except for the rabbit, have a keratinized buccal mucosa limiting their use as animal models. Rabbits possess a para-keratinized mucosa also hindering their effectiveness as an animal model. Ultimately, the direction of this research is to find a formulation capable of treating diabetes by Ins delivery through the buccal mucosa with adequate pharmacokinetics and we believe that the research and findings reported here contribute significantly to that goal.

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Vita

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